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VENOUS THROMBOEMBOLISM AND MECHANISMS DURING ASSISTED REPRODUCTIVE TECHNOLOGY

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Stockholm 2020

Published by Karolinska Institutet.
Printed by US-AB Tryckeriet.

ISBN 978-91-8016-018-6

Venous thromboembolism and mechanisms during assisted reproductive technology

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Friday December 11, 2020, at 9.00 a.m. Digital link found at KI webpage.

Till Mika och Tristan med moster Annas favorit:

To see a World in a Grain of Sand And a Heaven in a Wild Flower,
Hold Infinity in the palm of your hand And Eternity in an hour.

- William Blake -

ABSTRACT

Background

Assisted reproductive technology (ART) is increasingly used worldwide to treat infertility and assist couples and single mothers with their conception. The most common ART procedure includes treatment with exogenous hormones to stimulate the ovaries to produce multiple oocytes, followed by an in vitro fertilisation (IVF) achieved by letting oocyte and sperm combine *in vitro* or by direct intracytoplasmic sperm injection (ICSI) into the oocyte. After cultivation into an embryo for two to three days or five to six days into a blastocyst, a subsequent embryo transfer (ET) is performed, either a fresh ET or a frozen-thawed embryo or blastocyst transfer in a later, natural or ”programmed” cycle.

During pregnancy the incidence of venous thromboembolism (VTE) is at least five-fold increased as compared to same-aged, non-pregnant women and the incidence is even further increased during ART pregnancies. A doubled incidence has been reported during the whole pregnancy, mostly accounted for in the first trimester by a four-fold increased incidence of overall VTE and a seven-fold increased incidence of pulmonary embolism (PE) in women giving birth after ART as compared to matched women with spontaneous pregnancies. Even though the incidence is low with 1-2 per 1,000 in spontaneous pregnancies and 3-6 per 1,000 in ART pregnancies there has to be an awareness of the risk. VTE and PE are responsible for long-term morbidity, with the post-thrombotic syndrome and impaired exercise capacity after PE, and also an increased mortality with PE being a leading cause of maternal mortality. Since the highest incidence was found during the first trimester of ART pregnancies we hypothesised that this could be related to the hormone increase during ovarian stimulation.

Aims

The aim of this doctoral project was to achieve more knowledge of potential mechanisms behind the increased incidence of VTE found in ART pregnancies as compared to spontaneous pregnancies.

Methods and results

To gain knowledge on potential mechanisms explaining the prothrombotic state during ART, we analysed previously collected blood samples from 31 women undergoing ovarian stimulation. The samples were taken at down regulation of the ovaries, when endogenous oestrogen levels are almost undetectable, and during ovarian stimulation with its 10-100-fold increased oestrogen level. We aimed to detect changes of cell-derived plasma microvesicles (MVs) and found a significant increase of potentially prothrombotic MVs with

a majority of platelet-MVs exposing markers of activation and inflammation. Further, we analysed alterations of the plasma MV proteome to probe quantitative and qualitative changes of the whole MV protein content during ovarian stimulation. We aimed to capture and identify potential pathophysiological mechanisms concomitant with the oestrogen surge. We further aimed to find potential biomarkers that could help to identify the development of a hypercoagulative state that could help to explain the increased VTE incidence found in ART.

Furthermore, we performed a nationwide cohort study of all pregnancies during 20 years in Sweden and studied the incidence of VTE in women giving birth after a fresh ET and after a frozen, thawed ET performed in a later cycle. We compared these VTE incidences to that of women with spontaneous pregnancies. We found that the incidence of overall VTE and PE was more than eight-fold increased during the first trimester in the group of women with a fresh embryo transfer as compared to spontaneous pregnancies. We found no cases of PEs in the frozen ET group during the first trimester.

Conclusions

Our results could speak in favour of frozen ET over fresh ET to achieve a minimised risk of VTE after ART. The increase in MVs indicated a procoagulant state and platelet activation during the ovarian stimulation phase of ART. Larger study populations are required to evaluate whether the proteins identified in the MV proteome could have a potential as biomarkers of increased VTE-risk.

LIST OF SCIENTIFIC PAPERS

I. **Olausson N**, Mobarrez F, Wallén H, Westerlund E, Hovatta O, Henriksson P. Microparticles reveal cell activation during IVF - a possible early marker of a prothrombotic state during the first trimester. *Thrombosis and Haemostasis*. 2016;116:517-523.

II. **Olausson N**, Mobarrez F, Zubarev R, Chernobrovkin A, Rutishauser D, Bremme K, Westerlund E, Hovatta O, Wallén H, Henriksson P. Changes in the plasma microvesicle proteome during the ovarian hyperstimulation phase of assisted reproductive technology. *Scientific Reports*. 2020;10:13645.

III. **Olausson N**, Discacciati A, Nyman A I, Lundberg F, Hovatta O, Westerlund E. Wallén H N, Mobarrez F, Bottai M, Ekbom A, Henriksson P. Incidence of pulmonary and venous thromboembolism in pregnancies after in vitro fertilization with fresh respectively frozen-thawed embryo transfer: Nationwide cohort study. *Journal of Thrombosis and Haemostasis*. 2020;18:1965-1973.

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
APC	Activated protein C
ART	Assisted reproductive technology
AT	Antithrombin
AVK	Anti-vitamin K
BMI	Body mass index
CD40L	CD40 ligand
COC	Combined oral contraception
CRP	C-reactive protein
CT	Computed tomography
CTEPH	Chronic thromboembolic hypertension
CTPA	Computed tomography pulmonary angiography
DOAC	Direct oral anticoagulant
DR	Down regulation
DVT	Deep vein thrombosis
E1	Oestrone
E2	Oestradiol
E3	Oestriol
EMV	Endothelial microvesicle
ET	Embryo transfer
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
HLS	High level stimulation
HMWK	High-molecular-weight kininogen
HR	Hazard ratio
HRT	Hormone replacement therapy

ICMART	International committee monitoring assisted reproductive technologies
ICSI	Intracytoplasmic sperm injection
IRR	Incidence rate ratio
IVF	In vitro fertilisation
LC	Liquid chromatography
LH	Luteinising hormone
LMWH	Low-molecular-weight heparin
MBR	The Swedish Medical Birth Register
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MV	Microvesicle
NPR	The National Patient Register
OHP	Overall haemostasis potential
OHSS	Ovarian hyperstimulation syndrome
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PCOS	Polycystic ovary syndrome
PE	Pulmonary embolism
PMV	Platelet-microvesicle
PPV	Positive predictive value
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PTS	Post-thrombotic syndrome
SHBG	Sex hormone-binding globulin
TAFI	Thrombin activatable fibrinolysis inhibitor
TAT	Thrombin-Antithrombin
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
tPA	Tissue plasminogen activator

TRAP-6	Thrombin receptor activating peptide-6
VTE	Venous thromboembolism
vWF	von Willebrand factor

CONTENTS

1 INTRODUCTION

1.1 VENOUS THROMBOEMBOLISM

Venous thromboembolism (VTE) is the third most common cardiovascular disease after coronary heart disease and stroke and is caused by a combination of genetic and acquired risk factors. The term VTE includes deep vein thrombosis (DVT) and pulmonary embolism (PE). DVT most commonly occurs in the lower extremities in the valves of the calf veins, but can also occur at more unusual sites, such as the deep veins of the upper extremities, the abdominal or in rare cases even the cerebral veins. When parts of a thrombus loosen and travel with the circulation to another location of the vessel they are called emboli. The thrombi and emboli cause obstructions of blood flow. Around two thirds of all VTE manifest as DVTs and one third as PEs.

PE is considered to most commonly originate from DVTs of the lower extremities, but can also have its origin in the other mentioned localisations, such as the upper extremities, renal veins, inferior cava vein or the right atrium of the heart, see figure 1. PE restrict pulmonary arterial circulation and cause a variety of symptoms, e.g. dyspnea and pleuritic chest pain. Large emboli to the main or central pulmonary arteries can cause total blockage of the pulmonary circulation and subsequent hemodynamic collapse and cardiac arrest.

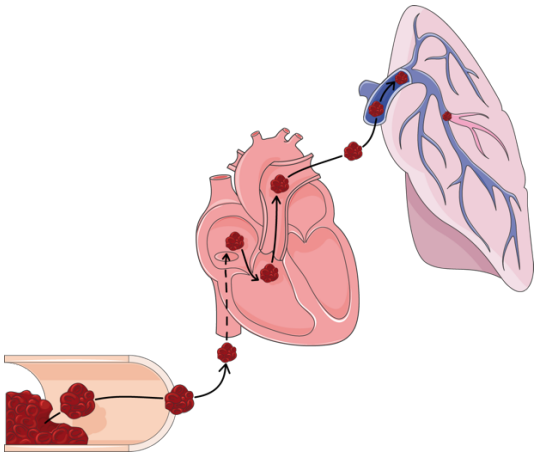


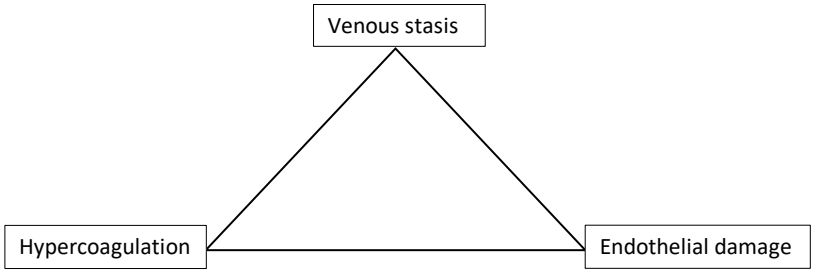
Figure 1. Pulmonary embolism. *Picture from Servier Medical Art licensed under the Creative Commons Attribution 3.0 Unported License.*

The incidence of VTE is reported to be around 1 per 1,000 annualy in the general population, while there is an increase with age and a difference between the sexes at different ages [1] [2]. In women below age 50, during the years of reproduction, the incidence of VTE is increased compared to men, but overall men have a higher risk than women both of first and recurrent VTE [3] [4]. Besides of age other strong risk factors for VTE are previous episodes of VTE with a recurrence risk exceeding 30 % within 10 years, family history and immobilisation after surgery [5]. Around 20 % of all VTE occur in cancer patients [6].

1.1.1 Virchow's triad

In the 19th century the pathologist Virchow summarised the risk factors for VTE in the three cornerstones still valid today and it includes the dysfunctional or damaged endothelium, blood components causing hypercoagulability and restriction of blood flow, venous stasis, as seen in figure 2 [7].

Figure 2. Virchow's triad of risk factors for thromboembolism.



The factors are often intertwined and they interact. Endothelial damage typically occurs after surgery, trauma, indwelling vein catheters, delivery and in cancer. A previous VTE can cause a long-term dysfunctional endothelium possibly contributing to a quite substantial risk of recurrence.

Venous stasis occurs when blood flow is restricted due to immobilisation after major ortophedic surgery in particular, hospitalisation after both surgery and medical illness, oedema due to heart failure or nephrotic syndrome, cancer with compression of the veins by tumours or during pregnancy with a growing uterus or the hormonal effect on the veins. Hospitalisation within three months after surgery or medical illness has been accounted for more than 50 % of all VTEs in an American study [6]. Immobilisation during long travel flights exceeding six hours entails a certain risk of VTE, in particular for travellers with other VTE risk factors [8].

Hypercoagulability can be related to both hereditary and acquired factors. Testing for hereditary thrombophilia in general include testing factor V Leiden gene mutation causing resistance to activated protein C (APC), the prothrombin gene mutation causing elevated levels of coagulation factor II and the gene mutations causing deficiency of the coagulation inhibitors antithrombin (AT), protein C and protein S. Thrombophilia testing has not been shown to reduce recurrent VTE and thus screening patients with a first event is not a routine in most clinics [9]. Family history alone is a strong indicator of VTE risk with a two to four-fold increased risk of a first VTE [10]. Other genetic risk factors are blood groups other than blood group 0 [11].

Among other than the mentioned acquired risk factors causing hypercoagulability are myeloproliferative disease, inflammatory disease and the antiphospholipid syndrome.

Still many cases of VTE are idiopathic, i.e. occur without a known risk factor. If a family history is present or when a need for individual risk assessment is desirable, thrombophilia screening can be warranted, but is still debatable [12]. To what extent cancer screening should be performed in patients experiencing a VTE without a known risk factor or symptoms is also debated. Studies have found an an incidence of cancer during the first two years after a first incident VTE 7-8 % [13 14].

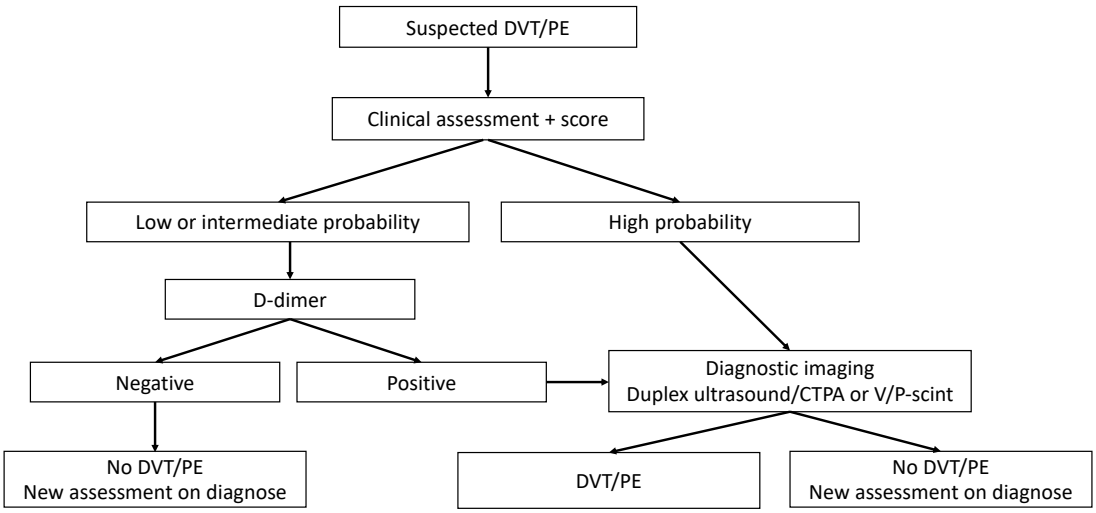
1.1.2 Diagnosing VTE

When diagnosing DVT in the lower extremities or suspected PE in hemodynamically stable patients clinical probability scores based on symptoms and risk factors should be used. Depending on pre-test probability and factors contributing to the risk of a false test result laboratory testing can be performed with the fibrin degradation product D-dimer. If D-dimer-testing is positive in a low- or intermediate risk patient or when the pre-test probability is high, diagnostic imaging will be requried. In suspected DVT of the lower extremitites duplex ultrasound with venography doppler and vein compression will be performed, while in suspected PE either a spiral computed tomography pulmonary angiography (CTPA) or a ventilation-perfusion (V/P) scintigraphy will be performed. Each hospital or care center should have an algorithm based on available methods, see figure 3. In some cases with a negative D-dimer a repeated duplex ultrasound has to be performed after a week, further diagnosing of the more proximal veins using CT angiography or MRI and a new assessment of other diagnoses that could cause the symptoms.

V/P-scintigraphy can be used when PE is supected in a hemodynamically stable patient. It could also be warranted when radiation to the female chest should be minimised, such as in pregnancy, in younger patients without pulmonary disease or when the presence of renal disease requires caution with contrast.

MRI could be another option in diagnosing VTE and there are ongoing studies on both PE and DVT. A previous study from 2010 on iliac vein thrombosis during pregnancy assessed duplex ultrasound and MRI in all 27 patients at pregnancy week 29 (range week 23-39) and in 3 women the iliac vein thromboses were detected only by MRI, thus missed diagnose with ultrasound [15]. Regarding recurrent DVT of the lower extremities recent studies suggest MRI to distinguish acute from chronic thrombi [16].

Figure 3. Diagnostic algorithm for DVT of the lower extremities and PE.



Prediction and diagnosis of VTE is important and adequate thrombembolic prophylaxis should be prescribed for patients at risk. Prediction scores and local guidelines on prophylaxis might be helpful. This was recently realised during the outbreak of the Coronavirus disease, Covid-19, where critically ill patients were found to have a high incidence of VTE despite standard thromboprophylaxis [17]. In severe Covid-19-infection markedly elevated D-dimers and fibrinogen levels are found indicating a hypercoagulable and hyperinflammatory state [18]. Several local guidelines now recommend to consider increased thromboprophylaxis in patients with severe Covid-19 infection and there is a lot of ongoing research on the etiology of the prothrombotic state in Covid-19 with continuously updated guidelines, thus not referred herein.

1.1.3 Morbidity and mortality in VTE

VTE is indeed a rare event, but bears a substantial risk of long-term complications and though rare even potentially fatal consequenses [2]. A Norwegian study on patients 20 years or older reported a case fatality rate of around 10 % for PE and 5 % for DVT with a doubled risk of dying from PE compared to DVT within 30 days after a first incident VTE [3]. Other studies reported an even higher case fatality rate within the first 3-12 months after diagnose of VTE ranging between 11-13 % [19 20]. A recently published study found a decreasing trend in the reported mortality rates for PE over the last 15 years in the United States with a mortality rate decreasing linearly from 12.8 (95 % CI 11.4-14) in 2000 compared to 6.5 per 100,000 (95 % CI 5.3-7.7) in 2015 [21].

Around 20-50 % of patients experiencing a DVT will develop a post-thrombotic syndrome (PTS) of varying severity with long-term complications including symptoms such as swelling, pain and heaviness of the legs after a DVT of the lower extremities [22-25]. A more rare complication after PE is chronic thromboembolic pulmonary hypertension (CTEPH) [26-28]. Many patients do also experience limitations in exercise capacity after PE without objective findings of CTEPH [29].

1.1.4 Treatment of VTE

Anticoagulant treatment of VTE today comprise novel or direct oral anticoagulants (DOACs) directly inhibiting thrombin or factor X, as the drugs of choice. Though in some conditions anti-vitamin K (AVK) is still recommended, for example in women breastfeeding or when repeated presence of antiphospholipid antibodies are found in patients in the antiphospholipid syndrome. AVK can also be considered in patients with massive VTE or VTEs at unusual thrombosis sites, such as cerebral veins or abdominal veins. During pregnancy subcutaneous injections of low molecular weight heparins (LMWH) are used instead of oral anticoagulants. LMWH could also be used when a bleeding risk is present or in situations when oral medication is not suitable or possible.

Most patients with a DVT can be treated as outpatients with start of DOACs at diagnosis. Proximal, ileofemoral DVTs with major symptoms can be considered for local, catheter-based thrombolytic treatment and thrombus removal. Regarding patients with PE, a risk stratification has to be performed to decide the therapeutic management. PE is a heterogenous condition ranging from low-risk PE with outpatient treatment using DOACs, to high-risk PE with hemodynamic instability or cardiogenic shock, requiring systemic thrombolytics to survive.

1.2 ASSISTED REPRODUCTIVE TECHNOLOGY

Assisted reproductive technology (ART) is increasingly used worldwide to facilitate reproduction. The term ART refers to interventions that include techniques handling egg (oocyte) and sperm to assist reproduction, in accordance with the glossary of the International committee monitoring assisted reproductive technologies (ICMART) [30]. It is estimated that more than 9 million children have been born after ART since the first child was born after IVF in 1978 in England [31]. In Sweden today around 4 % of all child births are conceived by ART [32]. ART treatment offers the possibility of conceiving a child with own or donated oocytes and sperm for couples or single women. Around 10-15 % of all couples are considered having fertility problems and with the increased age of parenthood and increased accessibility to the technology the number of children born after ART will most likely continue to increase in the future.

The most common procedure of ART is to use follicle stimulating hormone (FSH) to stimulate the ovaries to produce multiple oocytes for retrieval and subsequent fertilisation, while gonadotropin releasing hormone (GnRH) analogues are administered to suppress and hinder premature ovulation. The fertilisation is performed by either mixing the oocyte and sperm on a plate, standard IVF, or with the sperm directly injected into the oocyte, intracytoplasmic sperm injection (ICSI). The embryo is then cultivated for two to three days or up to five to six days to achieve a blastocyst before embryo transfer (ET) or cryopreservation is performed. The latter is performed using improved freezing methods with vitrification of the embryos at the blastocyst stage [33]. In many countries, such as Sweden, the standard recommendation of the National Board of Health and Welfare is to perform a single ET because of the increased pregnancy complications noted after a multiple pregnancy [34 35]. In the last yearly report of ART in Sweden every fourth ET leads to the birth of a child [36].

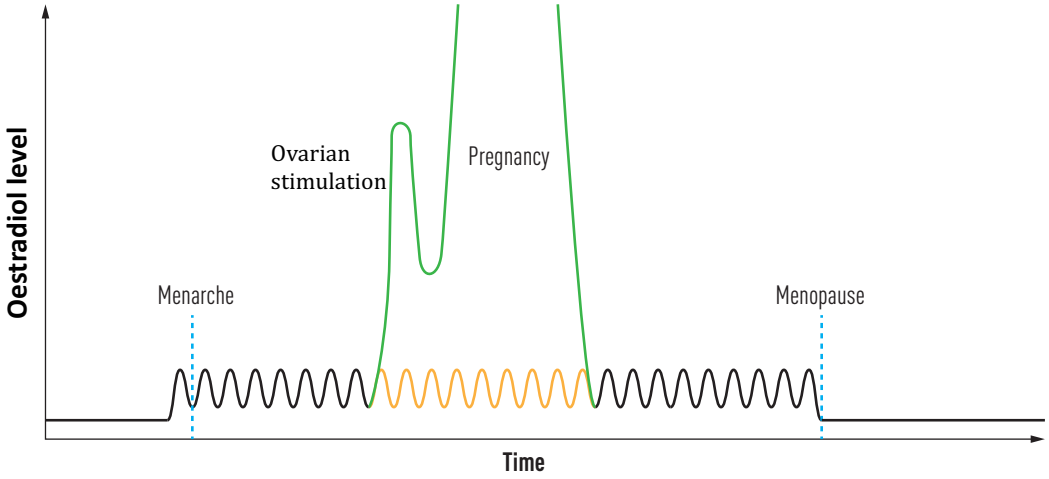
1.3 SEX HORMONES AND ENDOGENOUS OESTROGEN

The sex hormones are regulated by the axis of the hypothalamus-pituitary gland and the gonads. The hypothalamus produces GnRH, which signals to the anterior pituitary gland to release the gonadotropins, FSH and luteinising hormone (LH), which in turn signals to the ovaries and regulates the menstrual cycle.

The menstrual cycle starts with the bleeding, followed by the follicular phase with rising FSH levels with the aim to end in ovulation. Thereafter the luteal phase continues with proliferation of the endometrium to prepare for implantation, stimulated by progesterone secreted from the corpus luteum, the rest part of the follicle that has ovulated. If fertilisation does not occur the levels of oestradiol and progesterone decrease and induce a menstrual bleeding. When a woman is at fertile age, endogenous oestrogen, oestradiol E2, and oestrone E1, are mainly produced and secreted from the ovarian granulose cells, but also from the adrenal glands and in fat tissue, while oestriol E3, is produced by the placenta during pregnancy, see oestradiol levels in figure 4.

In a typical menstrual cycle the E2 level is increasing during the follicular phase with the highest level before LH surge which leads to ovulation. After the ovulation the progesterone levels are at the highest as mentioned to prepare the endometrial lining of the uterus for implantation. In a normal menstrual cycle the peak E2 reaches levels up to 1,500 pmol/L at maximum just before ovulation and then drops to below 200 pmol/L during the menstrual period. The level at menopause is even lower and many times not detectable [37]. During pregnancy a study on 52 women with uncomplicated pregnancies, E2 levels were measured with median levels 3,200 (SE±250) pmol/L, 16,000 (SE±960) pmol/L and 23,000 (SE±1,500) pmol/L in the third trimester of women with normal or low levels after one year with E2 <367 (SE ±55) pmol/L postpartum [38].

Figure 4. Oestradiol levels. Reprinted with permission from publisher [39].



ART offers a possibility to study the effects of supraphysiological oestrogen levels as induced by ovarian stimulation and lasting up to pregnancy week 8 after a fresh ET [40]. However, the wide range of E2 levels makes predictions and treatment effects of different E2 levels hard to compare [41]. In our previously published data on study population in study I-II, mean E2 level presented with a large spread with a mean of 5,889 pmol (SD±4,723 pmol/L) at high level stimulation [42].

Though, when E2 levels were divided into different categories with higher and lower levels, several studies have been able to correlate E2 to different outcomes. A positive correlation was found with E2 levels above 4,000 pg/mL, (corresponding to 15,000 pmol/L) at the day of hCG-administration, and pregnancy rate in women undergoing ART with ovarian stimulation [43]. In line with these findings another study found that low E2 levels early in ART pregnancies was associated with poorer pregnancy outcome [44]. High E2 levels are considered markers of OHSS risk, although the wide spread makes risk prediction difficult. A mean value of >3,500 pg/mL (corresponding to about 13,000 pmol/L) in OHSS without specifying severity grade, was found in a majority of studies according to a review by the American society for reproductive medicine [45].

There are various studies on endogenous oestrogens and risk of VTE. The hypothesis is that the longer the endogenous oestrogen exposure the higher the risk for VTE. In postmenopausal women oestrogen exposure was studied and exposure time defined by age at menopause and parity and they found that late menopause and multiparity was associated with an increased risk of VTE [46]. Women undergoing ART with ovarian stimulation are exposed to high levels of endogenous oestrogen and with a subsequent pregnancy with its high oestrogen levels a hypercoagulative state might be even more prominent. A Swedish study on ART and ovarian cancer or borderline ovarian tumours found an increased incidence of ovarian cancer in women who had given birth after ART as compared to spontaneously

conceived women [47]. Lifetime effect of ART on VTE incidence will have to be further studied in the future.

Exogenous oestrogens in combined oral contraception (COC) and in hormone replacement therapy (HRT) are associated with an increased risk of VTE [48]. Even in men with oestrogen treatment for prostate cancer an increased risk of VTE was found [49]. The VTE incidence in women using COC is three to six-fold increased in different studies and do also depend on which progesteron is used, administration route and oestrogen dose [50]. The incidence was increased by increased oestrogen dose [51] and depended on administration route, where oral oestrogens entailed a higher risk than transdermal [52]. The possible explanation is that the first pass hepatic metabolism induces effects in the liver that is a producer of most of the coagulation factors. Postmenopausal women with HRT have a three-fold increased risk of VTE found in the American study, The heart and estrogen/progestin replacement study [53]. It has been found that oestrogen has an immediate effect on haemostasis towards a hypercoagulable state as shown in a study of haemostatic factors measured 2-48 hours after intake of emergency contraceptive pill [54].

1.4 HORMONE PROTOCOLS IN OVARIAN STIMULATION

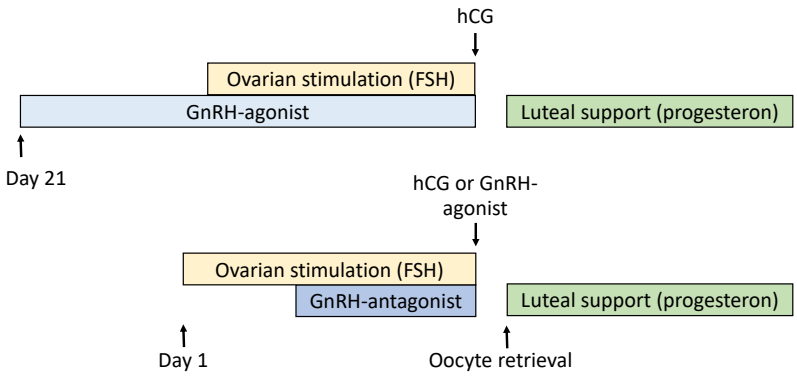
In ART ovarian stimulation with the gonadotropin FSH is used in order to achieve multiple follicles and oocytes for the fertilisation. FSH is given for about 10-14 days with monitoring of ovaries and uterus by ultrasound controls and sometimes oestradiol levels to assess follicular size, to adjust FSH dose and to decide on the timing of hCG to trigger the final oocyte maturation before oocyte retrieval.

In Sweden a shift from long agonist to short antagonist protocols took place in the beginning of the millennium and today mostly short protocols are used, see figure 5. This is due to the combined reason of a shorter treatment duration with a less amount of exogenous hormones and at a lower cost, see figure 5. The long agonist protocols have been associated with more oocytes retrieved and a higher pregnancy rate, but are also considered to increase the risk of ovarian hyperstimulation syndrome (OHSS) described below [55]. The shorter treatment protocols start with direct stimulation during the first day or days of the menstrual cycle with FSH, followed by a GnRH-antagonist after a few days to suppress ovulation. In these protocols a GnRH-agonist could be given instead of hCG to induce ovulation for example in women at risk for OHSS.

In the long protocol a GnRH agonist is started around day 21 of the menstrual cycle to down regulate the pituitary gland and the oestrogen level and to prevent the LH surge. After 10-14 days after a menstrual bleed or when the oestrogen level confirms DR, ovarian stimulation is started with FSH for around eight to ten days. Thereafter, human chorionic gonadotropin (hCG) is used to induce the final oocyte maturation. It acts similarly to the luteinising hormone (LH), but is a slower trigger so that oocyte retrieval can be scheduled after 36 hours. The GnRH agonist normally induces a short, initial raise in pituitary gonadotropins and

oestradiol level, but soon the pituitary is suppressed and hormone levels down regulated, followed by a drop in oestradiol and often a menstrual bleeding.

Figure 5. The hormones used in the long agonist protocol starting day 21 of the menstrual cycle and the short antagonist protocol starting in the first days of the menstrual cycle.



Ultrasound is used to monitor follicular development, often repeatedly in all protocols and treatment is individualised and monitored for dose adjusting and early signs of OHSS. When two to three follicles have reached the size over at least 16-18 mm and oestradiol levels, when measured indicate enough follicles, the hCG (or GnRH-agonist) can be administered for final maturation and oocyte retrieval. Oestradiol levels measure, as a role of thumb, around 700-1,000 pmol/L per follicle, and thus with 10 mature follicles the E2-levels would be measured to be at a desired level of around 10,000 pmol/L.

Progesterone is started after oocyte retrieval and continued until the pregnancy test, and is often administered vaginally.

1.5 FRESH EMBRYO TRANSFER

When an embryo is transferred to the uterus directly following ovarian stimulation and embryo cultivation, it is here referred to as a fresh ET. The remaining embryos are in the majority subjected to freezing strategies using vitrification during the freezing procedure. In most ART treatments a fresh ET will first be performed and thus, still more than half of all ETs are fresh ETs in most European countries, including Sweden [31]. If a woman develops OHSS or is at risk to develop OHSS a freeze-all embryos strategy should be used. Randomised clinical trials have shown similar or even higher pregnancy rates in frozen as compared to fresh ET [56 57].

1.6 FROZEN EMBRYO TRANSFER

When a frozen embryo is thawed and transferred to the uterus this is called a frozen-thawed ET or shortly, a frozen ET. The frozen ET can either be performed in a natural cycle, if the

woman has a regular menstrual cycle, or in an artificial menstrual cycle as induced by an equivalent regimen to that in HRT, mostly using low doses of exogenous oestrogen and subsequent progesterone administration after ultrasound control of endometrial thickness.

The timing of frozen ET is determined by measurement of LH in the woman's urine and when the LH-surge is detected and ultrasound confirms ovulation, the timing of the ET is determined. The timing is dependent on how many days that the embryo will be cultivated to match embryo age with the days after ovulation. The timing of the frozen ET can as mentioned also be regulated by hormone treatment, i.e. a "programmed" cycle.

1.7 OVARIAN HYPERSTIMULATION SYNDROME

OHSS occurs in moderate form in a few percent of all ovarian stimulation cycles and mostly early after hCG administration, but can also occur later in a subsequent pregnancy when endogenous hCG is released from the placenta. Mild forms of OHSS are probably underestimated and are presumed to occur in around 20-30 % of all treatment cycles, while moderate to severe form has been reported in 2-6 % of cycles [58 59]. OHSS is a syndrome with cystic enlargement of the ovaries and a leakage of fluid to the abdominal space around the ovaries due to increased vascular permeability. This peritoneal fluid causes abdominal pain, nausea and vomiting.

OHSS has been associated with a risk of both arterial and venous thromboembolism with reports of a remarkably high risk of VTE as compared to that in spontaneous pregnancy [60]. The pathophysiological mechanisms behind OHSS are still unclear, but the vascular permeability is engaging the endothelium. Some of the risk factors for OHSS are the polycystic ovary syndrome (PCOS), young age, low weight, previous OHSS, a history of elevated response to gonadotropins and multiple follicles retrieved (>15) [61 62].

1.8 VENOUS THROMBOEMBOLISM IN PREGNANCY

Pregnancy is a risk factor for VTE. During pregnancy and postpartum all cornerstones of Virchow's triad are active. The growing uterus causes mechanical compression of the iliac veins and inferior vena cava in the so called May-Thurner phenomena or iliac vein compression syndrome, where the right iliac artery or the ovarian artery crosses the left iliac vein and causes local disturbance. Furthermore, the hormonal changes during pregnancy with progesterone affect the veins with decreased vascular tone and dilatation of veins with stasis of the blood flow that might contribute to endothelial dysfunction and development of DVT in locations with decreased blood flow [63].

The incidence of VTE during pregnancy is 1-2 per 1,000 with the highest incidence in late pregnancy and the period after delivery, the postpartum period also called the puerpurium. The incidence of VTE is around five-fold higher than in non-pregnant women of the same

age and in the postpartum period the incidence has been reported to be 10-20-fold increased [64-68]. VTE and specifically PE is one of the leading causes of maternal mortality in the developed world with a mortality rate of 1 per 100,000 and the numbers in Sweden are consistent with these with 0.4-1 per 100,000 pregnancies [69 70].

It is more common during pregnancy to experience a DVT in the left leg, due to the May-Thurner phenomena described above. More proximal DVTs with iliac or iliofemoral vein thromboses are also more prevalent during pregnancy than in the population at general, with an increased risk of embolisation [71]. The distribution of DVTs versus PEs in pregnancy reveal a higher prevalence of DVT than in the general population with 80 % DVT and 20 % PE, the latter including simultaneous DVTs during pregnancy [72]. During pregnancy specific risk factors for VTE are multiple births, parity, caesarean section and preeclampsia [73].

Decisions on clinical probability of VTE in pregnancy can be demanding and the consideration of diagnostic options as well. The high cardiac output during late pregnancy can make it hard to diagnose PE with CTPA, with a risk of false positives with pulmonary arterial contrast defects mistaken for PE or false negatives when peripheral emboli could be missed out. V/P scintigraphy can in turn be difficult to interpret. Radiation to both the foetus and the female chest should preferably be minimised. Therefore, a bilateral ultrasound of the lower extremities and iliac veins could sometimes be performed instead of pulmonary arterial imaging in suspected PE, given that the patient is hemodynamically stable [74]. Unfortunately it can also be difficult to examine and visualise an isolated iliac vein thrombosis, especially in late pregnancy. Thus, the previously mentioned MRI-study performed in Gothenburg found that MRI was an option in pregnancy. New scoring systems including adjusted D-dimer levels are under development, but not enough validated as to date [75].

1.9 VENOUS THROMBOEMBOLISM IN ASSISTED REPRODUCTIVE TECHNOLOGY

A Norwegian population-based case-control study of risk factors for pregnancy-related VTE found that ART was a risk factor for VTE in women giving birth [76]. They proceeded this study with a hospital-based case-control study of 559 cases of VTE during pregnancy and two non-pregnant controls for each case, where they also found that ART was a risk factor with an additive effect of multiple births [77]. Previous to these studies there had been indications of VTEs in ART in numerous case reports and case series on ART with upper extremity and subclavian vein thromboses related to OHSS, with a possible publication bias for the unusual thrombosis sites [78 79].

Since then population-based studies have been performed aimed to study the incidence of VTE after ART and table 1 shows a summary of the incidences and risk ratios or hazard ratios reported in these four larger, registry-based studies and a smaller, prospective cohort study.

Table 1. Studies of assisted reproductive technology and venous thromboembolism.

Author, year	Rova et al. 2012	Henriksson et al. 2013	Hansen et al. 2014	Villani et al. 2015	Filipovic-Pierucci et al. 2019
Study design	Nationwide cohort study	Nationwide cohort study	Nationwide cohort study	Regional cohort study	Nationwide cohort study
Study population	19,194 women giving birth after ART in Sweden 1999-2008 935,338 controls spontaneous pregnancies	23,498 women giving birth after ART in Sweden 1990-2008 116,960 matched controls spontaneous pregnancies	16,191 women giving birth after ART in Denmark 1995-2005 819,751 controls all pregnancies including ART	234 women with a clinical pregnancy after ART in Italy 2002-2011 3,339 controls spontaneous pregnancies 2010-2012	82,174 women giving birth after ART in France 2013-2015 Control group spontaneous pregnancies
VTE in ART, incidence (n)	5.9 per 1,000 (114)	4.2 per 1,000 (99)	2.8 per 1,000 (48)	8.5 per 1,000 (2)	3.3 per 1,000 (107)
VTE in control group, incidence (n)	1.5 per 1,000 (1428)	2.5 per 1,000 (291)	1.2 per 1,000 (727)	1.8 per 1,000 (6)	Not reported
Risk ratio or similar	**Antepartum OR 2.7** 95% CI 2.1-3.6 **Postpartum OR 1.2** 95% CI 0.6-2.0	**HR 1.8** 95% CI 1.4-2.2	**IRR 1.7** 95% CI 0.9-3.0 **Antepartum: IRR 3.0** 95% CI 2.1-4.3	**OR 3.9** 95% CI 0.87-15	**IRR 1.2** 95% CI 1.0-1.3 (including ovarian induction in ART-group)
First trimester VTE in ART, incidence (n)	1.7 per 1,000 (32)	1.5 per 1,000 (36) PE: 0.3 per 1,000 (7)	0.62 per 1,000 (10)	Not reported	Not reported
First trimester VTE in control group, incidence (n)	0.17 per 1,000 (160)	0.3 per 1,000 (38) PE: 0.04 per 1,000 (5)	0.11 per 1,000 (90)	Not reported	Not reported
Risk ratio or similar	**OR 9.8** 95% CI 6.7-14	**HR 4.1** 95% CI 2.5-6.5 PE: **HR 7.0** 95% CI 2.2-22	Single birth **IRR 5.9** 95% CI 2.7-13 Multiple birth **IRR 8.0** 95% CI 2.5-25.5	Not reported	**IRR 3.3** 95% CI 2.2-4.8 (only ovarian stimulation)

ART=assisted reproductive technology, VTE=venous thromboembolism, PE=pulmonary embolism, n=number, HR=hazard ratio, OR=odds ratio, CI=confidence interval, IRR=incidence rate ratio.

Hansen *et al* published a study in 2012 including all ART treatments with ovarian stimulation and compared the incidence of VTE to that of a control group of women of the same age not using oral contraception, and found no difference [80]. In their second study in 2014 shown in table 1, they analysed only the ART treatments leading to a child birth, including 18,787 women compared to a reference group of spontaneously conceived women [81]. They found an increased risk of VTE all over, in particular during the first trimester and in the postpartum period and in multiple birth. Further, they presented in 2018 a study of the VTE incidence during the first 12 weeks after ART with early pregnancy loss before 10 completed

pregnancy weeks [82]. They found a low incidence, only one case, incidence 1.3 (95 % CI 0.03-7.49) per 10,000 pregnancies and compared to IVF with completed pregnancy 7.8 (95 % CI 4.1-13.3) per 10,000 (RR 0.17, 95 % CI 0.02-1.3).

Rova *et al* published a population-based cohort study in 2012 of 19,194 women giving birth after ART during a 10-year period 1999-2008 compared to a control population of women with spontaneous conception during the same period [60]. They found an incidence of 1.7 per 1,000 in ART with an almost 10-fold increased risk during the first trimester after ART compared to all other deliveries during the same period, 0.17 per 1,000 (odds ratio, OR 9.8, 95 % CI 6.7-14). They also reported that 6-7 % of all women that were hospitalised due to OHSS had a 100-fold increased incidence during the first trimester after fresh ET. They reported no difference after frozen ET.

In 2013, Henriksson *et al* published a population-based study on 23,498 women conceived after their first ART pregnancy, compared to 116,969 women, matched on age and calendar period, giving birth to their first child [83]. They found a doubled incidence of VTE, 4.2 per 1,000 in women conceived by ART respectively 2.5 per 1,000 in women naturally conceived (hazard ratio, HR 1.8, 95 % CI 1.4-2.2). The incidence was in particular increased during the first trimester with a seven-fold increased incidence of PE 3.0 per 1,000 versus 0.4 per 1,000 (HR 7.0, 95 % CI 2.2-22) and for first trimester VTE 1.5 per 1,000 versus 0.3 per 1,000 (HR 4.1, 95 % CI 2.5-6.5). The distribution between DVT and PE was consistent with previous studies, 80 % DVT and 20 % PE.

Villani *et al* performed a prospective cohort study comparing VTE incidence of 234 women who had a clinical pregnancy after ART to a reference cohort of women who gave birth in the same region, spontaneously conceived in 2002-2011 [84]. They found a VTE incidence of 8.5 per 1,000 compared to 1.8 per 1,000 in controls, (OR 3.9, 95 % CI 0.87-15.3) with this small sample thus no significant result. OHSS was found in 4.3 % of the ART population, of which none developed a VTE though only 30 % received thromboprophylactic treatment. They found thrombophilia in 10.3 % of the women in the ART group and one of those experienced a VTE.

Hansen *et al* in 2012 found, as mentioned, no association to VTE after unsuccessful ART. In a French study by Filipovic-Pierucci *et al*, they did find such an association studying fertility treatments in a nationwide study in France in women undergoing fertility treatments including ART with ovarian stimulation [85]. They studied 788,007 treatment cycles resulting in 82,821 deliveries in 82,174 women during the years 2013-2015 and compared the incidence of both venous and arterial thromboembolism in different treatment groups to a control group of non-pregnant women and women with a spontaneous pregnancy respectively. Interestingly as mentioned, they found an association between ovarian stimulation not leading to pregnancy and VTE, with an incidence rate of 3.4 per 10,000 person-years (95 % CI 1.2.0-5.5) and as compared with a non-pregnant control group an incidence rate ratio (IRR) of 1,76 (95 % CI 1.31-2.37). In the group of women who delivered after ART with ovarian stimulation they found an incidence of 3.3 per 1,000 compared to

spontaneous pregnancies IRR 1.32 (95 % CI 1.08-1.62), adjusted for age and multiple births compared to spontaneous pregnancies.

From the RIETE- registry, an ongoing multi-centre study collecting real world data on VTE cases in different centres since 2001, an observational study was conducted on VTE events in women of childbearing age during 2001-2016, which revealed 6,718 VTEs of which 41 were ART-related VTE. The events of VTE were as many in absolute numbers in ART leading to pregnancy (n=20) as in unsuccessful ART (n=21). Most of the VTE occurred in the first trimester, 14 out of 20 events (70 %) [86].

In summary, there is an association between ART and the incidence of VTE in women giving birth after ART and a particular risk in OHSS. There is still a need of further development of the ART treatment and prophylactic treatment recommendations.

1.10 HAEMOSTASIS

Haemostasis is the process when the body reacts on a bleeding with a response to minimise blood loss. The system is strictly regulated in order to prevent excessive clot formation with a close interaction between endothelium and the circulating blood components. When the endothelium is injured there is an immediate response of vasoconstriction, which is followed by platelet adhesion and aggregation forming the initial platelet plug. When further repair is necessary or endothelium activation continues further activation of both the platelets and the coagulation cascade occurs to generate thrombin and to form fibrin networks stabilising the plug. When the system is in disorder it can cause thromboembolism or bleeding problems.

As soon as a clot is starting to form to repair the injured vessel there is also a process of inhibition of excessive clot formation and dissolution of the clot through fibrinolysis.

1.10.1 Primary haemostasis

The injured or dysfunctional endothelium exposes adhesion factors such as collagen and von Willebrand factor (vWF), which bind to both the subendothelium and to platelets that thus adhere to the vessel wall and form the initial platelet plug. The platelets will be activated and undergo major shape change, shifting their negatively charged inner membrane of phospholipids to the outside and develop long pseudopodia that facilitate further adhesion and aggregation.

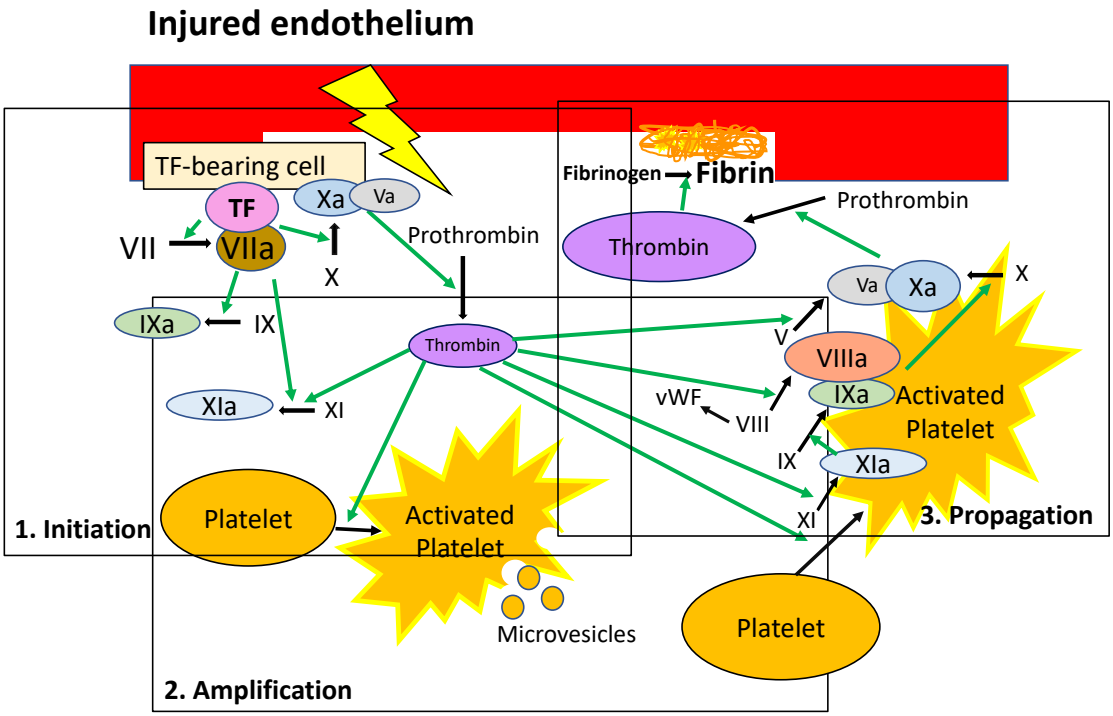
The activated platelets secrete a number of prothrombotic proteins, including adhesion proteins and coagulation factors, from their granules and expose these proteins on their own surface. This further activates and facilitates platelet aggregation and adhesion to endothelial cells and leukocytes. The activated platelets also bud off small vesicles termed microvesicles (MVs). These platelet-MVs (PMVs) are thus both markers of platelet

activation and also act procoagulant since they, like their parent cell, provide a negatively charged phospholipid surface for the coagulation factors to assemble on and take part in amplification and propagation of coagulation with increased thrombin generation and fibrin formation, in the so called cell-based model of haemostasis.

1.10.2 The cell-based model of haemostasis and secondary haemostasis

The cell-based model of haemostasis describes the coagulation process in three phases, initiation, amplification and propagation and in the model the primary and secondary haemostasis are intertwined leading to the formation of a clot [87], see figure 6. The process includes the activated platelets and MVs that provide a surface for the coagulation factors to assemble on and multiply in the cascade of enzymatic reactions leading to thrombin generation and fibrin networks that form the clot.

Figure 6. The three phases of coagulation.



The main initiator is tissue factor (TF) exposed by the injured endothelium or by other TF-bearing cells such as monocytes or MVs. TF binds to and activates coagulation factor VII and the complex, TF-factor VIIa, activates factor X. Activated factor X (factor Xa) together with activated factor V (factor Va) then activate prothrombin (factor II), resulting in a small amount of thrombin (factor IIa).

In the amplification phase the small amount of thrombin generated further activates platelets at the site of injury and also activates factor V, factor VIII and factor XI.

In the propagation phase, TF-VIIa-complex activates factor IX to factor IXa, which together with factor VIIIa gather on the phospholipid surface of activated platelets or MVs in the presence of calcium and factor X. This complex of factor IXa-VIIIa, the tenase complex, rapidly activates factor X that also assembles on the platelet or MVs and forms the prothrombinase complex of factor Xa-factor Va in the presence of calcium.

Large amounts of thrombin are now generated that can cleave fibrinogen to fibrin monomers which are cross-linked and stabilised by factor XIII.

1.10.3 Regulatory processes

There are several regulatory processes involved in haemostasis of which some are mentioned. The initiation of coagulation with TF and factor VIIa is inhibited by the tissue factor pathway inhibitor (TFPI) by inhibiting activation of factor X. Thrombin as one of the most important coagulation factors, that converts fibrinogen to fibrin, activates other coagulation factors and platelets directly, needs to be inhibited through several mechanisms. AT is one of these important natural inhibitors of thrombin by forming the thrombin-antithrombin complex (TAT) and AT also inhibits factor IXa, factor Xa and factor XIa. AT is the target of anticoagulant treatment with low molecular weight heparins (LMWH). The third important inhibitor is APC which inactivates factor Va and factor VIIIa with protein S as a co-factor. This step is activated by thrombin binding to thrombomodulin on endothelial cells activating protein C. A deficiency of any of these three proteins AT, protein C or S entails an increased risk of developing VTE [88].

1.10.4 Fibrinolysis

As soon as a blood clot is formed fibrinolysis starts and degradation products, such as D-dimers are formed, that can be measured in plasma. Plasminogen is a protein bound to fibrin surfaces and it is activated by tissue plasminogen activator (tPA) to plasmin. The activated plasmin starts the dissolution of the clot and degradation products will be formed. The fibrinolytic process is regulated by plasminogen activator inhibitor-1 (PAI-1) and during pregnancy the placental plasminogen activator inhibitor-2 (PAI-2) and thrombin activatable fibrinolysis inhibitor (TAFI) in order to slow down fibrinolysis and prevent bleeding.

Thrombin is thus both a major activator of platelets and coagulation, converting fibrinogen to form fibrin, but also a regulator of anticoagulant and fibrinolysis-inhibiting pathways.

1.10.5 Microvesicles

One way of studying cell-based haemostasis and platelet function is to study changes of the procoagulant MVs. The most abundant plasma circulating MVs are the above mentioned platelet-MVs (PMVs) released from activated platelets. MVs can also derive from a variety of other cells, such as endothelial cells, leukocytes and erythrocytes following activation or apoptosis. MVs are small, membrane-coated, sized 0.1-1 μm in diameter and as mentioned they share the protein composition of their parental cell and carry surface proteins with various biological effects that can be transferred to other cells. MVs are thus important in transcellular signalling and communication and have been associated with both inflammatory and prothrombotic processes [89].

Studies of changes in MVs and their protein composition over time in disease or compared to healthy controls can reveal pathophysiological mechanisms in various diseases and MVs have been found elevated in both cardiovascular disease such as acute coronary syndrome [90], peripheral arterial disorder [91] and in diabetes [92]. The MVs procoagulant properties can be attributed to the surface layer of phospholipids with the phophatidylserine (PS) that provides the surface for the assembly of coagulation factors, but also through the exposure of TF and other procoagulant proteins [93 94 95].

Regarding VTE, many studies on MV levels in VTE-patients have been performed on cancer-patients, but there are also studies to be found on VTE in general. In a case-control study of 186 patients with a first VTE, they found increased levels of all PS-positive MVs, MVs of platelet-origin and MVs bearing TF analysed by flow cytometry compared to in 418 controls, adjusted for age, body mass index (BMI), sex and thrombophilia [96]. Another study compared MV levels as measured by flow cytometry in 25 VTE patients to 25 controls and found increased levels of endothelial-MVs (EMVs) [97]. In hereditary thrombophilia increased levels of PS-positive MVs, PMVs, EMVs and TF-bearing MVs have been found in carriers of factor V Leiden gene mutation [98], prothrombin gene mutation [99] as well as in AT-, protein C- and protein S-deficiencies compared to healthy controls [100]. In all thrombophilia studies they also found an association between carriers of the deficiencies of the natural coagulation inhibitors who had experienced a previous VTE and higher levels of MVs [100].

Platelet activation as measured by P-selectin and CD40 ligand (CD40L), both released from platelets upon activation, have been examined in thrombotic disease such as VTE and also in complicated pregnancies or spontaneous abortion. P-selectin has been associated with VTE in mouse models with decreased venous thrombosis when treated with P-selectin inhibitor [101]. In a study of 28 patients with a previous idiopathic DVT an association was found between soluble P-selectin and the VTE-patients compared to age-and sex-matched controls [102]. In the same study they found no elevation of CD40L. In another study of 304 women with a previous VTE who had experienced at least one spontaneous abortion, they found that stillbirth after pregnancy week 24 was associated P-selectin level, but not with miscarriages before that week in women who had a history of a VTE [103]. The receptor for P-selectin, P-selectin glycoprotein ligand-1 (PSGL-1) has also been found increased as carried by

monocyte-MVs (MMVs) and EMVs in patients with a previous idiopathic VTE compared to in age-matched healthy controls [104]. P-selectin released soluble to the circulation or membrane-bound exposed on PMVs could both interact with leukocytes by binding to the PSGL-1 mostly expressed on leukocytes.

1.11 HAEMOSTATIC CHANGES IN PREGNANCY

The haemostasis changes during pregnancy towards a hypercoagulative state which contributes to the regulation of blood loss during delivery, though the uterine contraction at delivery is the most important factor to stop postpartum-bleeding. Uterine atony is one of the leading causes of maternal death in developing countries.

During pregnancy there is an increase of coagulation factors, especially factor VIII [105 106], factor X [105], factor XII [105], vWF and fibrinogen [107 106]. With a progressing pregnancy the coagulation inhibitors decrease. Antithrombin decreases in most studies, at least towards the end of pregnancy and still postpartum [108], but remains unchanged in some studies [109]. Protein S decreases [107 110] and APC-resistance increases [111] during the progress of pregnancy, while various studies have described protein C levels to both increase [112] and decrease or to remain unchanged [107 113].

All this together with an impaired fibrinolysis contributes to the hypercoagulative state that progresses during pregnancy along with the great increase in oestrogen level as indicated previously [114-116]. The levels of PAI-1 and naturally the placenta-produced PAI-2 increase later in the pregnancy [110 112]. Regarding tPA various data exist with some studies finding increased levels [107] and some decreased [117]. In one study of 48 uncomplicated pregnancies they found besides an increased PAI-1 and PAI-2 levels a decreased level of tPA throughout pregnancy [112], while another study of 41 women with uncomplicated pregnancies indicated a decrease of tPA during the first trimester followed by an increase in late pregnancy [118]. This was speculated to be due to an increased fibrinolysis during late pregnancy as a natural protection, a way to counteract the hypercoagulability. They found the same increase of PAI-1 and PAI-2 and all levels returned to pre-pregnancy values six weeks postpartum. Increased D-dimer through pregnancy suggests increased fibrinolysis as well as a hypercoagulative state. Other possible counteractors are the increased levels of the coagulation inhibitor TFPI that has been found to be increasing during pregnancy [119].

Other studies using global haemostasis assays have found increased coagulation parameters in TEG [120] and in ETP and peak thrombin as measured by the calibrated automated thrombogram (CAT) [109].

The most common findings in haemostatic parameters are presented in table 2. Regarding some of the factors, studies have shown varying results and some factors vary by trimester. Factor II and factor V have been found to be elevated during early pregnancy and to decrease

to normal levels by the third trimester [106]. Factor IX and XI have also been shown to both increase and decrease in the different trimesters and postpartum.

Factor XIII that stabilises the clot has mostly been found to be decreased in pregnancy but there are conflicting data. In one study they thus assessed more parameters simultaneously, such as clot strength as measured by thromboelastography (TEG), fibrinogen and platelet counts simultaneously and repeatedly during pregnancy and compared these values at 8 weeks postpartum as a reference, with different time points during pregnancy to assess possible relations [121]. They found that factor XIII activity decreased during pregnancy, while platelet counts decreased and fibrinogen increased and both were positively correlated to clot strength, while factor XIII activity was not.

Platelet aggregation has recently been assessed in women giving birth with repeated blood sampling during pregnancy and postpartum and compared to the postpartum samples and to a control group of fertile women, they found a minor decrease in platelet aggregation during pregnancy compared to postpartum and to healthy, non-pregnant controls [122]. Platelet activation and aggregation was induced by arachidonic acid, adenosine diphosphate (ADP), collagen and thrombin receptor activating peptide-6 (TRAP-6).

In pregnancy PMVs and EMVs have been found elevated compared to in non-pregnant women [123]. MVs in uncomplicated pregnancies have been found elevated in the first trimester compared to healthy, non-pregnant controls and the MV levels increased with gestational age [124].

Sex differences in MV levels have been investigated and in a study of premenopausal women compared to age-matched men [125]. The women were found to have elevated PS-positive MVs with PMVs expressing P-selectin, detected by flow cytometry. The difference was largest during the women's luteal phase.

In recently menopausal women MVs were assessed by flow cytometry in two groups as divided by E2-level [126]. Twenty-one of the women had low oestradiol levels (E2 ≤20 pg/ml corresponding to around ≤70 pmol/L) and 11 of the women had higher levels (E2>40 pg/ml corresponding to around >150 pmol/L). PS-positive MVs and MVs exposing TF as well as all other MVs measured, derived from endothelial cells, monocytes, and granulocytes and were significantly increased in the group of women with low endogenous oestrogen.

1.12 HAEMOSTATIC CHANGES IN ASSISTED REPRODUCTIVE TECHNOLOGY

Most studies on haemostasis during ovarian stimulation have found that the procoagulative parameters increase concomitant with a decrease in natural anticoagulants and an impaired fibrinolysis with a close similarity to what is found during pregnancy.

In table 2 the changes are presented during pregnancy respectively during ART.

Increased levels have been found of coagulation factors II, VIII, IX [127 128], von Willebrand factor [127] and increased fibrinogen [127 129]. Factor VII has remained unchanged [129] or with decreased levels [127]. Factor V have been found decreased [130].

Westerlund *et al* performed studies on global haemostasis in women undergoing ovarian stimulation, which is also the study population of study I and II in this thesis. They assessed with the CAT assay the endogenous thrombin potential (ETP) and analysed also overall haemostasis potential (OHP) and found an increased thrombin generation as exemplified by ETP and an increased fibrin formation by the OHP method [42].

Regarding coagulation inhibition TFPI has been found to decrease [130 131]. APC-resistance increased towards late pregnancy [132]. Decreased levels have been found of AT [127 129 130 133], protein C [127] as well as protein S in one study [132], while the latter had a tendency to increase in another [127].

Furthermore, as in other pregnancies an impaired fibrinolysis has been found during pregnancy with decreased levels of tPA [134], but PAI-1 also decreased in this study while PAI-2 increased.

MV studies during ART are scarce. In a study of recurrent implantation failure in women conceived by ART by a Spanish research group, it was noted increased MV levels following recurrent implantation failure after ART as compared to in those with successful ART treatment and to fertile controls [135].

Table 2. Haemostatic parameters during pregnancy and ART

Haemostatic parameter	**Change in ART**	**Change in pregnancy**
Coagulation factors		
Fibrinogen	↑	↑
Factor II	↑	various
Factor V	↑	various
Factor VII	various	various
Factor VIII	↑	↑
Factor IX	↑	↑
Factor X	-	↑
Factor XI	-	various
Factor XII	-	↑
Factor XIII	various	↓
von Willebrand factor	↑	↑
Coagulation inhibitors		
Antithrombin	↓	↓
Protein C	↓	various
Protein S	various	↓
APC-resistance	↑	↑
Fibrinolytic factors		
tPA	↓	various
PAI-1	various	↑
PAI-2	↑	↑
Cell-derived microvesicles		
Microvesicles (MV)	-	↑
Platelet-derived MV (PMV)	-	↑
MV exposing P-selectin	-	↑
MV exposing tissue factor	-	↑
Leukocyte-derived MV	-	↑
Endothelial-derived MV	-	↑
Global haemostatic assays		
Thromboelastography	↑	↑
Overall haemostasis potential	↑	↑
Endogenous thrombin potential	↑	↑

2 AIMS

The overall aim of this thesis was to gain increased knowledge of the association between assisted reproductive technology (ART) and venous thromboembolism (VTE) with focus on the effects of ovarian stimulation and its hyperoestrogenism as a potential cause of the procoagulative state and increased VTE-risk found in ART after ovarian stimulation.

The specific aims of the three studies were:

- Study I: To study the effects of ART with ovarian stimulation on circulating plasma microvesicles exposing procoagulative and inflammatory markers.

- Study II: To explore the changes of the protein composition (proteome) of the plasma microvesicles during ovarian stimulation in ART to identify physiological and potential patophysiological mechanisms that could potentially contribute to a prothrombotic state.

- Study III: To assess the incidence of VTE and PE in women giving birth after ART using fresh embryo transfer, when the transfer was performed directly after the ovarian stimulation cycle, or when a frozen-thawed embryo transfer was performed in a later cycle, and compare to the incidence of VTE and PE after ET to that in women giving birth after spontaneous conception.

3 MATERIALS AND METHODS

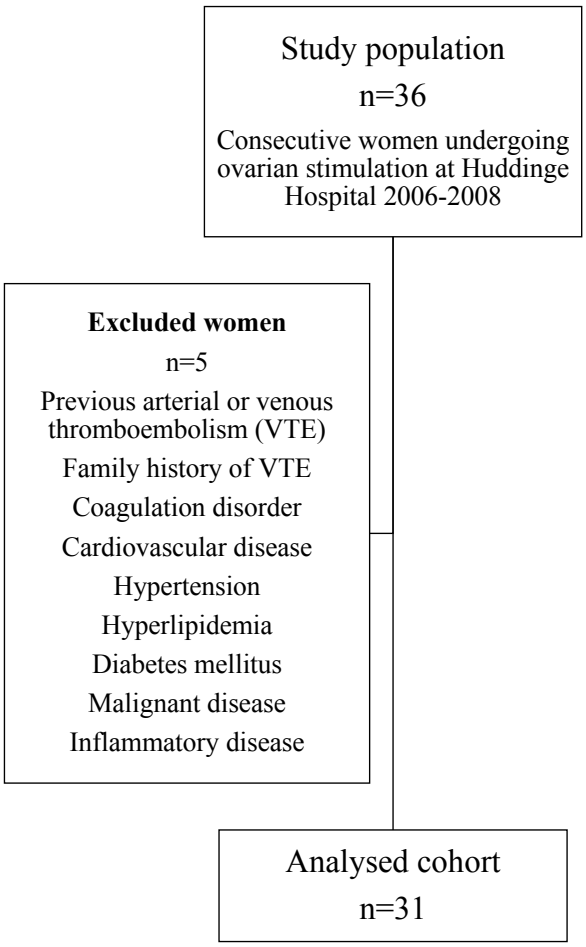
3.1 STUDY POPULATION AND STUDY DESIGN

3.1.1 Study I and II – The microvesicle studies

3.1.1.1 Study population

The study population of study I and II consisted of 31 women undergoing ART treatment with ovarian stimulation at Huddinge Hospital in Stockholm, Sweden in 2008-2009, see figure 7. The women were consecutively included by Westerlund *et al* as previously described, unless they met the exclusion criteria: previous arterial or venous thromboembolism, family history of VTE, coagulation disorder, cardiovascular disease, hypertension, hyperlipidemia, diabetes mellitus, active or previous history of a malignant or systemic inflammatory disease [42].

Figure 7. Study population in study I-II, flow chart.



Some of the patient characteristics are presented in table 3.

Table 3. Characteristics of the 31 patients in study I and II.

Age, years	33.0 ± 3.3
BMI, kg/ m^2	24.1 ± 3.6
Smoking, n (%)	2 (11)
Female cause of infertility, n (%)	7 (22)
Male cause of infertility, n (%)	12 (39)
Unknown, n (%)	12 (39)
Standard IVF/ICSI/discontinued, n	21/9/1
Clinical pregnancy, n (%)	12 (39)
Ovarian hyperstimulation syndrome, n (%)	3 (9.7)

Age and BMI=body mass index presented as means and standard deviation. Other data in numbers and proportion of patients. n=number.

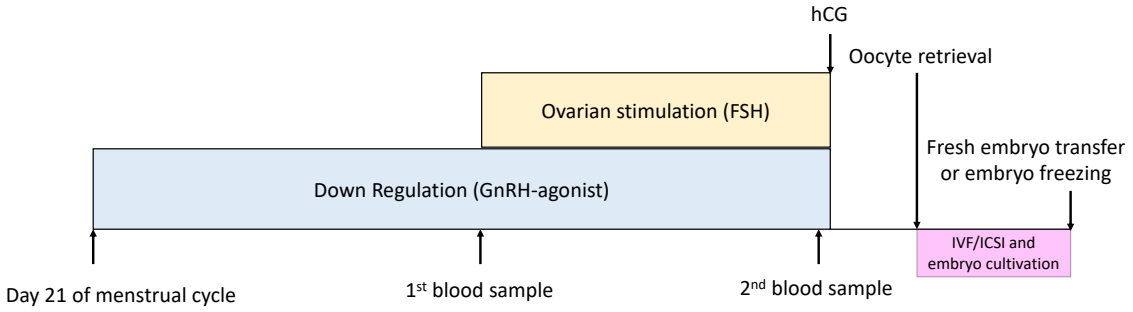
All women received the long hormone protocol using a GnRH-agonist, which was started on day 21 of the menstrual cycle with a nasally administered spray (Buserelin). Approximately two to two and a half weeks later a down regulation (DR) of the ovaries was achieved with oestradiol levels below 150 pmol/L, and thereafter the ovarian stimulation was started with subcutaneous injections of FSH (Gonal-F) administered for 10-14 days. Repeated ultrasound images of the ovaries were performed during the ovarian stimulation to regulate follicular development by dose adjustments and finally hCG was administered subcutaneously 36 hours before planned oocyte retrieval.

3.1.1.2 Blood sampling

Blood samples were collected at two occasions as shown in figure 8, through direct venepuncture under overnight fasting conditions and after 20 minutes of rest, sitting down. The first sample was taken two to two and a half weeks after treatment start, at maximum DR when oestradiol levels were <150 pmol/L and a menstrual bleed had occurred. The second sample was taken at high level stimulation (HLS) before hCG was administered.

The blood was collected in citrated tubes (containing nine parts of blood and one part of sodium citrate) and in EDTA-tubes. The samples were immediately centrifuged at 2000 g for 20 minutes at room temperature to obtain platelet poor plasma (PPP) and aliquots of 500 µL were freeze stored in -80º.

Figure 8. Treatment protocol in study I-II.



3.1.1.3 Study design

The studies were performed by analysis of blood samples before and during ovarian stimulation. Each patient served as their own control with comparisons of samples before and during ovarian stimulation.

3.1.1.4 Ethical approval and considerations

The studies were performed in accordance with the ethical principles of the Declaration of Helsinki for medical research. The studies were approved in 2006 by the Regional Ethical Review Board in Stockholm, Sweden, reference number 2006/1222-31/1. All patients gave their informed consent to participate in the study with a consent to store blood samples in the research biobank. They were all informed that they could demand to be excluded from the study at any time and of their right to ask for their individual data results and for their samples to be destroyed or unidentifiable.

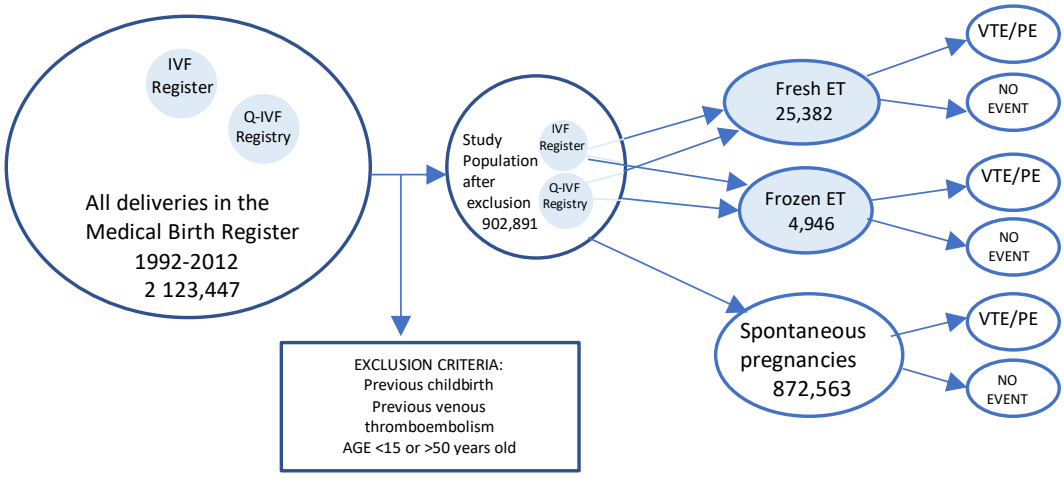
3.1.2 Study III – The observational study

3.1.2.1 Study population and study design

The study population in study III consisted of all deliveries from the Swedish Medical Birth Register (MBR) [136] in Sweden registered in 1992-2012, see figure 9. Up until 2006 information on ART was collected and presented as the IVF part of the registry and since 2007 collected by the National Quality Registry of Assisted Reproductive Technology (Q-IVF) [36]. Out of the women with child births during this period as identified in the MBR we sorted out the women that were found in the IVF-part of the MBR and in the Q-IVF which constituted a cohort of all ART pregnancies. In our study design we wanted to study women with first child births in all groups and the first incident outcome VTE or PE. Thus we excluded all women with a previous child birth and all women with a previous VTE. We also chose to include women who were 15-50 years old. A total of 30,328 women with a first delivery constituted all women after ART and they were further divided into the groups of 25,382 women who were conceived after fresh ET and the 4,946 women who had a frozen

ET. The rest of the pregnancies were considered as spontaneous pregnancies and numbered 872,563 women with a first delivery.

Figure 9. Study population in study III.



3.1.2.2 The registries

The registries used in this study were thus the MBR and the Q-IVF registry [36] for study population and further the National Patient Register (NPR) in Sweden for all diagnoses of VTE, the Swedish Register of Education [137] for information on completed years of education and the Swedish Cause of Death Register [138] for death in the postpartum period. All information in the registries mentioned are based on the unique Swedish identity number.

The MBR held at the National Board of Health and Welfare contains detailed information on 97-99.5 % of all child births in Sweden since 1973 with information obtained from prenatal, delivery and neonatal care and up until 2005 births from pregnancy week 28 were included and from 2005 and on from pregnancy week 22 [136]. As mentioned, information on all ART was included in the MBR from 1982 until 2006 with information concerning for example ART method and date of ET.

Since 2007 the Q-IVF collects information on all ART treatments in Sweden and not only deliveries. It contains detailed information, such as on treatment protocols used, dates of treatment start and of all other registered events, whether own or donated oocytes and sperm were used, fertilisation method (IVF or ICSI), number of embryos transferred and whether a clinical pregnancy was achieved or not [3].

The NPR started registering inpatient diagnoses in the 1960s, but it was not until 1987 that the reporting was made mandatory in Sweden on all in-patient care in the register that is held

by the National Board of Health and Welfare. In 1997 also outpatient diagnoses were introduced and in 2001 all specialist outpatient care are included. The coverage is considered high concerning the inpatient part of the registry and the validity is high for most diagnoses [139]. Regarding the outpatient diagnoses the first year is considered to be potentially underreported for many diagnoses. The diagnoses are coded according to the International Classification of Disease (ICD), which has been released in different versions and today since 1997-98 the ICD-10 is used in Sweden.

Statistics Sweden are responsible for the Education Register, which contains information such as the highest educational level completed.

3.1.2.3 Ethical approval and considerations

The study was performed in accordance with the ethical principles of the Declaration of Helsinki for Medical Research. Ethical approval was granted in 2009 by the Regional Ethical Review Board in Stockholm, Sweden, reference number 2010/267-31/4, and in 2013, reference number 2013/1849-31/2. All registry-data are as mentioned decoded on beforehand at the National Board of Health and Welfare and since the personal identity number is replaced with a serial number no individuals can be identified by the researchers or in the event of data intrusion, which is important since registry data does not require personal consent to be used in research.

3.2 METHODS AND STATISTICAL ANALYSIS

3.2.1 Study I – The microvesicle study

3.2.1.1 Microvesicle analysis

The frozen samples of PPP were thawed and centrifuged a second time during 20 minutes at 2,000 g in room temperature (RT). The supernatant was then re-centrifuged at 13,000 g for 2 minutes at RT and 20 µL of the supernatant was incubated during 20 minutes in the dark with phalloidin to exclude cell fragments as previously performed in our research group [140].

A flow cytometer (Beckman Coulter Gallios) was used to detect and quantify the MVs. The flow cytometry sorts the cells based on size, shape and fluorescence [141]. In the analysis cells pass through a laser beam in a solution and when the light falls on each cell it is spread or scattered in a certain manner depending on size and granularity, which allows for each cell to be determined. The side scatter of the light determines complexity or granularity, while size is determined by the forward light scatter and calibration beads of known sizes are used as a reference to compare size. In this study a mix of beads of the sizes 0.5, 0.9 and 3.0 µm was used.

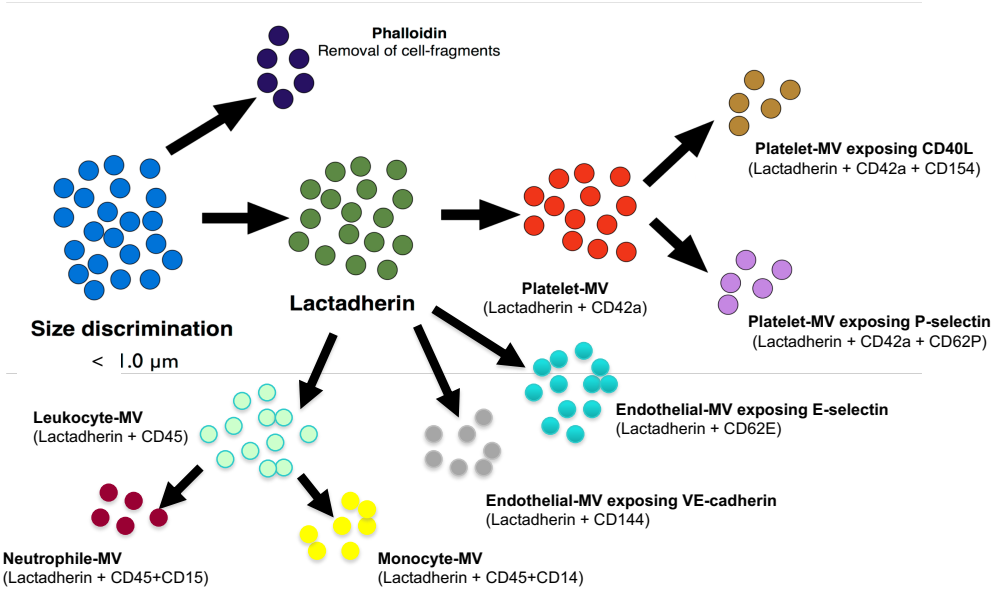
Annexin V or lactadherin are proteins used to detect the negatively charged phospholipid surface exposure. Lactadherin binds to PS without requiring calcium and in this study

fluorescence-labelled lactadherin was used to identify the total MV counts as PS-positive MVs. Further, specific fluorescence-labelled monoclonal antibodies were used to detect cell origin and activation markers.

MVs were defined as vesicles smaller than 1.0 µm, negative to phalloidin and positive to lactadherin, PS-positive, and further phenotyped by the chosen monoclonal antibodies in our study protocol. They were calculated as absolute numbers given in MVs/L by a formula of (MV count x standard beads/L)/standard beads counted.

Our MV protocol, seen in figure 10, aimed to determine all MVs as PS-positive MVs and further phenotype them into platelet-MVs (PMVs) as MVs positive for CD42a and PMVs with platelet activation markers CD62P for P-selectin, CD154 for CD40 ligand or CD40L, endothelial MVs (EMVs) as positive for CD62E for E-selectin or CD144 for VE-cadherin and monocyte-MVs (MMVs) as MVs positive for CD14 and for neutrophile-MVs (NMVs) as MVs positive for CD15.

Figure 10. Microvesicle protocol in study I. Revised image, original by Fariborz Mobarrez.



3.2.1.2 Statistical analysis of the microvesicles

The MV distribution showed a skewness to the right even after log transformation, which was both visually seen in the histogram and confirmed by Shapiro-Wilks test for normality. Thus, the statistical analysis was performed by non-parametrical tests.

Data is presented as medians and interquartile ranges (IQR) for the continuous variables such as microvesicle counts. Comparisons of the values before and during ovarian stimulation

were performed by Wilcoxon signed-rank-test for dependent data or matched pairs. The two-tailed significance level was set to 0.05. Sample size of at least 31 patients had been calculated in advance to achieve a desired power of at least 80 % power to detect a difference of 20 % between the samples at a 5 % significance level. Simple linear regression analysis was performed to test the relationship between variables. The flow cytometric analysis had previously been performed on MVs in healthy controls by repeated measurements on two different flow cytometers in different laboratories with intra- and interassay coefficient of variation below 10 %.

3.2.2 Study II – The microvesicle proteome study

3.2.2.1 Proteomic analysis of the microvesicles

Several proteomic techniques are available to detect and measure the abundance of proteins. In this study liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to determine the protein composition of MVs. The analysis was performed on the MV-enriched samples prepared, to identify and quantify changes of the proteins carried by the MVs during ovarian stimulation.

The proteomic analysis is complex with many steps, both in the laboratory setting and in the data processing with the use of several software programs that handle large scale bioinformatics and determine the protein by matching the unique peptide sequences of the analysed material with previously known data. In this study liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) was used according to the detailed, previous description in the published article [142].

The proteins or peptides of a sample need to be prepared before analysis, which was achieved by liquid chromatography (LC) after incubation with trypsin which digested the proteins into peptides. The peptides were then separated by LC analysis that was coupled to the tandem-MS analysis in order to identify and quantify the proteins through the unique peptides by the mass-to-charge ratio giving the abundance. Data was further analysed by software analysis for peptide identification of the raw data with a predetermined requirement of at least two unique peptides consisting of minimum length of 6 amino acids identified per protein and with a false discovery rate (FDR) of 1 %.

3.2.2.2 Statistical analysis of the proteomic analysis

The data was log-transformed due to skewness in the distribution and was confirmed to be normally distributed after log2-transformation. Comparisons of protein abundance before and during ovarian stimulation were assessed by moderated paired t-test using the limma package [102]. Proteomics and other studies on large scale data entail a risk of achieving a large number of significant results by chance due to the multiple comparisons that can lead to false positive and inaccurate interpretations. Thus, adjustment for multiple comparisons should be

performed. We used correction for the multiple comparisons using Benjamini-Hochberg adjustment of the false discovery rate (FDR) and set the significance level to 1 %, the adjusted P-value, often referred to as q-value less than 0.01.

3.2.3 Study III – The observational study

3.2.3.1 Registry data

Data was collected from the registries with the study population from the MBR as described above where all women were aged 15-50 years old with their first child born during the study period from the 1st of January 1992 until the 31st of December 2012.

The exposures were either pregnancies achieved by ART by a fresh ET or by a frozen ET and those were compared respectively to the reference group of spontaneous pregnancies. The reference group comprised the women found in the MBR that were not included in the IVF-registry part or Q-IVF and were thus considered to be spontaneous pregnancies.

The outcome VTE was defined as the first incident diagnose of VTE and secondary outcome was first incident PE during the time period. All women with a previous VTE were excluded.

For the outcomes the ICD-codes included were for **PE** (ICD-8: 450.01-03, 450.09, 673.98-99; ICD-9: 415B, 673C; ICD-10: I26.0, I26.9, O882), for **DVT** (ICD-8: 451.00, 451.98-99, 671.01-02, 671.08-09; ICD-9: 451B; ICD-10: I80.1-3, I80.8-9, O22.3, O87.1), portal vein thrombosis (ICD-8 453.09; ICD-9: 452; ICD-10: I81.9), vena cava thrombosis (ICD-8; ICD-9: 453C; ICD-10: I82.2), renal thrombosis (ICD-8; ICD-9: 453D; ICD-10: I82.3), cerebral vein thrombosis (ICD-8: 321.00, 321.09; ICD-9: 325; ICD-10: O22.5, O87.3) and diagnoses codes for other localizations of DVT or emboli (ICD-8: 453.09; ICD-9: 453W, 453X, 671F; ICD-10: I82.8-9, O87.9).

The follow-up period included time from the start of the pregnancy until an event occurred or until 42 days postpartum. Trimesters were determined as day 0-90 for first trimester, day 91-181 for second trimester and day 182 until three days before delivery date for third trimester, and two days before delivery date and 42 days after delivery for postpartum.

3.2.3.2 Statistical analysis

Incidences of the outcomes VTE and PE were calculated as events per 1,000 pregnancies with all antepartum and postpartum periods included.

Hazard ratios (HR) with 95 % confidence intervals (CI) were estimated by Cox regression models for the two different exposures, fresh and frozen ET and compared to spontaneous pregnancies, which was defined as the non-exposed or reference group. HRs were estimated for the entire period including all trimesters and the postpartum period.

We performed two models, where the first model calculated crude hazard ratios and the second model had adjustments made for the potential confounding factors: age at delivery (<25, 25-29, 30-34 or ≥35 years), pre-pregnancy BMI (<25, 25-29 or ≥30 kg/m^2), single or multiple birth, pre-pregnancy cigarette smoking, calendar period of diagnosis split into 5-year intervals (1992-1996, 1997-2002, 2003-2007, 2008-2012), educational level (≤9, 10-12 or >12 school years) and country of birth.

4 RESULTS AND DISCUSSION

4.1 STUDY I – THE MICROVESICLE STUDY

4.1.1 Results

In table 4 the previously analysed haemostatic parameters in the study population performed by Westerlund *et al* are presented [42]. As previously mentioned the oestradiol levels increased 10-100-fold from the down-regulated level at DR compared to during the final phase of the ovarian stimulation just before hCG-administration, HLS, as denoted above in the description of study population and design. Their findings showed increased fibrinogen, factor VIII and thrombin generation as assessed by ETP by the CAT assay [42 143].

Table 4. Baseline values and change in oestradiol and some haemostatic parameters. Reprinted with permission from publisher [144].

	DR Mean ± SD	HLS Mean ± SD	Reference interval	P-value
Age, years	33.0 ± 3.3			
BMI, kg/m^2	24.1 ± 3.6		< 25	
Haemoglobin, g/L	129.0 ± 8.6		117 - 153	
Leukocyte particle conc x10^9/L	5.1 ± 1.2		3.5 - 8.8	
Platelet particle conc x10^9/L	251.2 ± 47.6		165 - 387	
Creatinine, µmol/L	68.9 ± 7.3		< 90	
LDL-cholesterol, mmol/L	2.9 ± 0.7		1.4 - 4.7	
Plasma glucose, mmol/L	4.7 ± 0.5		4.0 - 6.0	
Oestradiol, pmol/L	106 ± 31	5,889 ± 4,723		<0.001
Fibrinogen, g/L	2.8 ± 0.7	3.3 ± 0.7	2.0 - 4.0	<0.001
FVIII, kIU/L	0.96 ± 0.34	1.26 ± 0.41	0.5 - 1.8	<0.001
Peak, nM IIa	290 ± 42	343 ± 38		<0.001
ETP, nM IIa*min (AUC)	1,542 ± 287	1,739 ± 288		<0.001

DR=down regulation, HLS=high level stimulation, SD=standard deviation, BMI=body mass index, LDL=low density lipoprotein, ETP=endogenous thrombin potential, AUC=area under curve. P-values were obtained by Student's t-test.

The medians of MV counts were calculated and presented with interquartile ranges (IQRs) and values at DR and HLS were compared.

MVs included in the analysis are shown in the protocol in figure 10. MVs were defined as those positive to lactadherin and further as PMVs when positive to CD42a, EMVs exposing E-selectin as positive to CD62E, EMVs exposing VE-cadherin as positive to CD144, MMVs as positive to CD14 and NMVs to CD15. Further, PMVs exposing activation markers P-selectin and CD40 ligand as positive to both CD42a and CD62P respectively CD154.

As seen in figure 11, we found a significant increase in most of the MVs analysed by the specific monoclonal antibodies according to our protocol.

The most abundant MVs were PMVs, which is a common finding in MV analyses and makes MV analysis a way of studying changes of platelet function and platelet activation. PMVs exposing P-selectin (CD62P+) and CD40 ligand (CD154+) were both significantly increased as seen in figure 12.

Further, we analysed EMVs as MVs exposing the adhesion molecules E-selectin and VE-cadherin, both adhesion molecules and indicators of endothelial activation. EMVs exposing E-selectin (CD62E+) were significantly increased, while EMVs exposing VE-cadherin (CD144+) remained unchanged. The only group of MVs that were slightly decreased where NMVs (CD15+).

In the study population three out of 31 (9.7 %) women were diagnosed to have developed OHSS and comparison of overall PS-positive MV levels in these three women to the rest of the women showed that overall MVs were four-fold increased as compared to the other 28 women, with a tendency towards significance (p=0.072).

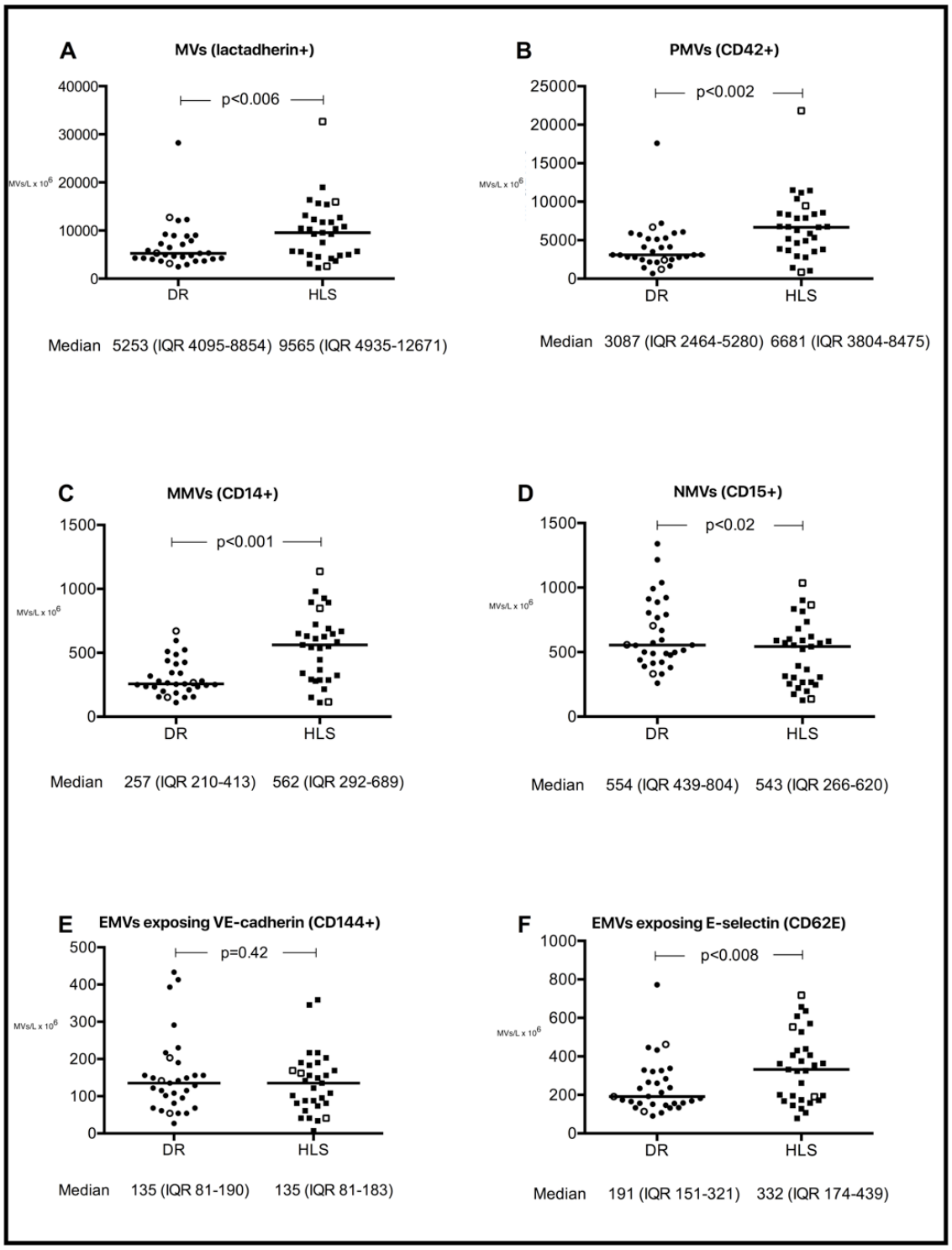
To assess correlations between the variables we performed simple linear regression analysis. No associations were found between oestradiol levels and MV levels. The material was then divided using oestradiol levels by a median split, to those with values above and below the median. We then performed a regression analysis of the previously analysed haemostatic parameters and found a correlation between oestradiol levels above the median at HLS and peak thrombin at HLS.

4.1.2 Discussion

We found that the levels of circulating PS-positive MVs increased during ovarian stimulation indicating a procoagulant state with an increased amount of phospholipid surfaces available for coagulation.

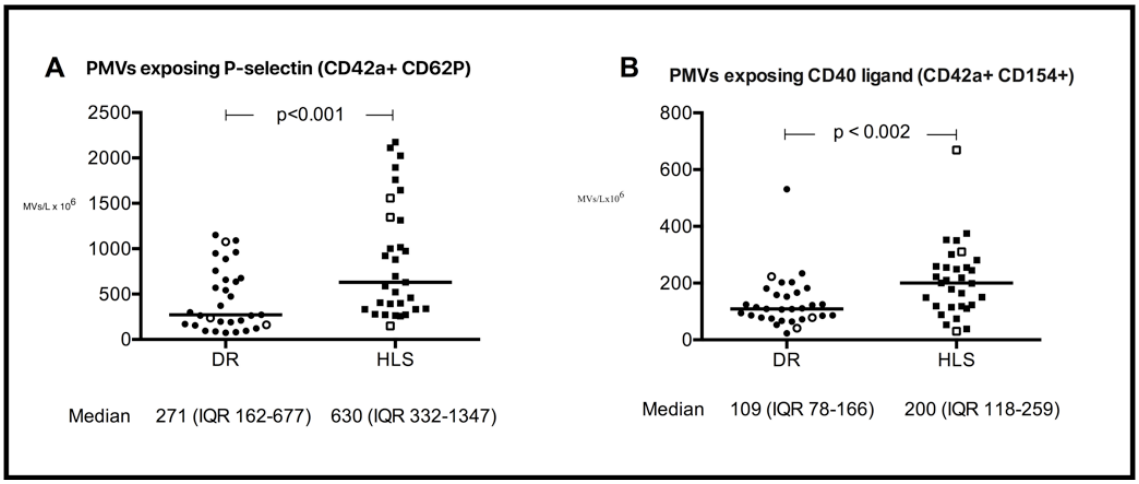
PMVs were the most abundant and increased significantly and PMVs exposing P-selectin and CD40L doubled during ovarian stimulation indicating platelet activation and an increased possibility of cross-talk between inflammation and haemostasis since they are both released from the platelet granules upon activation and can bind to receptors on other cells such as leukocytes and endothelial cells.

Figure 11. Microvesicles before (DR) and at (HLS) ovarian stimulation. Reprinted with permission from the publisher [144].



DR=down regulation, HLS=high level stimulation, IQR=interquartile range, MVs=microvesicles, PMVs=platelet microvesicles, MMVs=monocyte microvesicles, NMVs=neutrophile microvesicles, EMVs=endothelial microvesicles

Figure 12. Platelet-microvesicles exposing activation markers P-selectin and CD40 ligand MVs before (DR) and at (HLS) ovarian stimulation. Reprinted with permission from the publisher [144].



DR=down regulation, HLS=high level stimulation, IQR=interquartile range, PMVs=platelet microvesicles

Of the leukocyte-derived analysed MVs, the MMVs increased, while NMVs decreased slightly. EMVs exposing E-selectin increased, while EMVs exposing VE-cadherin remained unchanged [104]. There are studies that have found a stronger association to apoptosis in VE-cadherin [145], which could suggest that ovarian stimulation causes endothelial activation but not apoptosis in endothelial cells.

Platelet activation, as indicated by increased amount of both soluble P-selectin and MV-bound P-selectin, has been investigated in pregnancies complicated by preeclampsia and in pregnancies with spontaneous recurrent abortion compared to uncomplicated pregnancy and non-pregnant controls. In one study of PMVs exposing P-selectin in preeclampsia they found that the levels were significantly increased in preeclampsia compared to the non-pregnant controls, while there was no significant change as compared to normotensive pregnant controls [146]. All staining for P-selectin also stained for the platelet-specific monoclonal antibody (CD61) suggesting that most P-selectin reflect platelet activation. In preeclampsia the platelet numbers decreased, which in turn decreased overall PMVs, yet the fraction exposing P-selectin was as mentioned increased. In two studies on of P-selectin during pregnancy the women who developed preeclampsia had significantly elevated levels at the end of the first trimester, suggesting that P-selectin could be a marker of complicated pregnancy with preeclampsia [147] [148].

CD40L can be measured both as soluble CD40L and MV-exposed CD40L. Soluble CD40L levels have been associated with inflammatory and thrombotic diseases with worse outcome in acute coronary syndrome [149]. In contrast to our findings, CD40L measured in soluble form by ELISA decreased compared to baseline in women during ovarian stimulation at the day of

hCG-administration, followed by increased levels at the day of oocyte retrieval [150]. The oestradiol mean levels in that study were very similar to those in our study population (5,572 ± SE 3,296 pmol/L) and CD40L seemed to decrease by increasing oestradiol level, but with no significant correlation to the also found wide range of oestradiol.

MVs exposing CD40L have been suggested to be a better marker of inflammation than soluble CD40L. This was found in a study of 15 healthy patients with in vitro-induced inflammation and flow cytometric MV analysis of MV-exposed CD40L compared to soluble CD40L [151]. Soluble CD40L was found at significantly higher levels in preeclampsia compared to uncomplicated pregnancies, but interestingly with the same levels as the non-pregnant controls [152].

An interesting finding was that, though non-significant, the small number of women in our study who developed mild OHSS had a four-fold increased MV count as compared to the other 28 women. Whether MVs could be biomarkers of OHSS, which in turn could contribute to an increase of VTEs in ART pregnancies, needs to be further studied.

4.1.2.1 Limitations and methodological considerations

One limitation of our study is the small sample size, but the study design with each patient being her own control in the pre-during ovarian stimulation design reduced the risk of confounding that could otherwise exist between patients. The design requires fewer patients to detect changes with statistical significance, though a statistically significant result might not always be of clinical relevance.

Another limitation of the method is that the detection limit is 0.2-0.3 μm and small MVs could be missed. Also we could interpret other particles such as lipoproteins or other small vesicles as MVs.

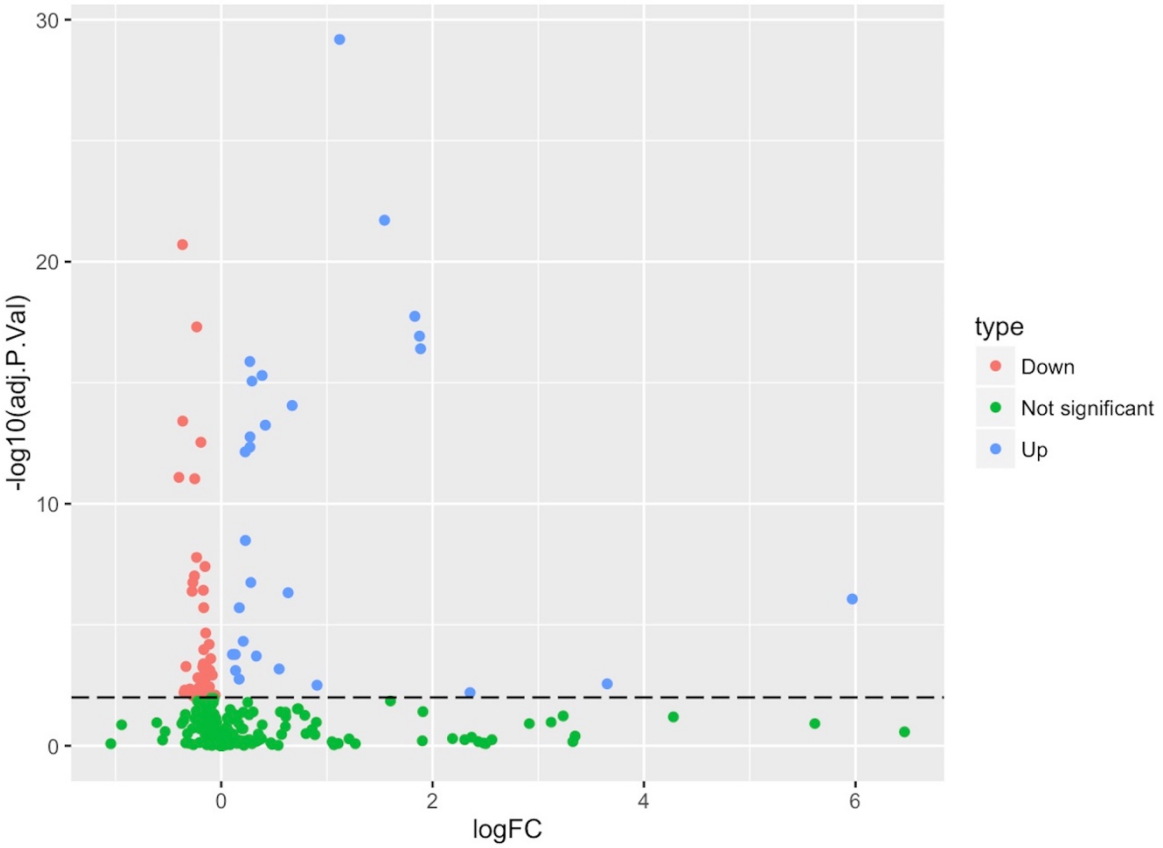
In this study we did not perform any functional assay of procoagulant properties of the MVs, but it has previously been shown that MVs are procoagulant by the expression of PS and that the thrombin generating capacity of PS-positive MVs was high and decreased with blocking of PS by lactadherin [91 153].

4.2 STUDY II – THE MICROVESICLE PROTEOME STUDY

4.2.1 Results

In total 1,199 proteins were initially identified by LC-MS/MS and further analysis was performed on all proteins that were quantified in more than 50 % of the samples as the lower limit of detection. Thus, 306 proteins were included in the analysis with comparison of protein abundance before and during ovarian stimulation, that is at DR and at HLS and they are plotted out in the Volcano plot, see figure 13.

Figure 13. Volcano plot of all analysed proteins (n=306) and their change during ovarian stimulation. The x-axis represents the logarithm to the base 2 of the fold change (FC) and the y-axis the negative logarithm of the adjusted P-value. Dotted line demarks the significance level <0.01. Reprinted with permission from the publisher [142].



In the Volcano plot in figure 13 the changes of proteins are shown with the log 2 of the fold change. As previously described, P-values were corrected using Benjamini Hochberg method for multiple comparisons. We identified 72 proteins that were significantly changed in abundance at HLS as compared to DR, with a significance level <0.01. Out of the 72 proteins, 28 were up-regulated and 44 down-regulated during ovarian stimulation. In table 5 all the identified, known proteins are categorised, but since many proteins have several biological functions and because those are intertwined the proteins often belong to more than one of the groups stated in the table.

Table 5. Significantly changed proteins ($p<0.01$)

UP-REGULATED	DOWN-REGULATED
Coagulation factors	
Fibrinogen alpha chain	Coagulation factor V
Fibrinogen beta chain	
Fibrinogen gamma chain	
von Willebrand factor	
Other proteins involved in coagulation	
Fibulin-1	Histidine rich glycoprotein
Kininogen-1	
Vitronectin	
Coagulation inhibitors	
	Antithrombin
	Protein S
Fibrinolytic components	
	Alpha-2-antiplasmin
	Tetranectin
Complement components	
	Complement C1q subunit A
	Complement C1q subunit B
	C4-binding protein alpha chain
	C4-binding protein beta chain
	Complement factor B
	Complement factor I
	Complement factor C4B
	Complement component C6
	Complement component C7
	Complement component C8 gamma chain
	Plasma protease C1 inhibitor
Acute phase proteins and proteins involved in inflammation	
Alpha-1-antitrypsin	Alpha-1-antichymotrypsin
Angiotensinogen	Alpha-1-acid glycoprotein 1
Alpha-2-HS-glycoprotein	Alpha-1-acid glycoprotein 2
Ceruloplasmin	Haptoglobin
	Ig gamma-1 chain C region
	Ig lambda chain V
	Ig lambda-2 chain/Ig lambda 3-chain
	Immunoglobulin J chain
	Monocyte differentiation antigen CD14
	N-acetylmuramoyl-L-alanine amidase
Proteins involved in reproduction	
Fetuin-B	
Pregnancy zone protein	
Sex hormone-binding globulin	
Lipoproteins	
Apolipoprotein A-I	Apolipoprotein A-II
	Apolipoprotein B
	Apolipoprotein C
	Apolipoprotein D
	Apolipoprotein E
	Serum-Amyloid A-4 protein
Cytoskeletal proteins	
Actin	Gelsolin
Heat shock cognate 71 kDa protein	Proteoglycan 4
78 kDa glucose-regulated protein	
Miscellaneous	
Carbamoyl-phosphate synthase	Cholinesterase
Corticosteroid-binding globulin	Extraceullar matrix protein 1
Hyaluronan-binding protein	Glutathione peroxidase
Hemoglobin subunit alpha	Transthyretin
Hemoglobin subunit beta	
Inter alpha trypsin heavy chain H4	
Lipopolysaccharide-binding protein	
Thyroxine-binding globulin	
Vitamin-D-binding protein	

4.2.2 Discussion

With proteomic analysis of the microvesicle proteome content we detected significant changes in haemostatic markers with up-regulation of several coagulation and complement-related factors during ovarian stimulation. Further, acute phase proteins acting for example in response to stress or infection, were altered.

Among the significantly changed proteins a large number of the up-regulated proteins were related to the coagulation and the complement cascade with up-regulated levels of vWF, fibrinogen and kininogen-1 that is a precursor of high-molecular-weight kininogen (HMWK) and down-regulation of AT and protein S.

Furthermore, several proteins serving as acute phase reactant proteins were also up-regulated indicating a pro-inflammatory state. Levels of the acute phase protein C-reactive protein (CRP) was estimated during ovarian stimulation in 28 women undergoing ART with ovarian stimulation and showed a high CRP during early ovarian stimulation, measured on day three of stimulation, that was associated with treatment failure [154].

Sex hormone-binding globulin (SHBG) is a main carrier of oestradiol in circulation and was up-regulated, which might be expected due to the increased oestrogen level during ovarian stimulation. SHBG has been studied as a potential risk marker for VTE during treatment with oral contraception. Increased levels of SHBG in COC-users have shown to correlate to increased APC-resistance and higher SHBG levels have been found in patients using COC that are associated with increased VTE-risk [155 156].

There are numerous proteomic studies of follicular fluid, oocyte and sperm aimed to predict fertilisation, but there is a lack of published studies on the plasma MV proteome during ovarian stimulation. We found proteins in our study that could be of interest in both studies of fertility and reproduction and regarding the risk of VTE.

Studying MVs and the MV proteome during ovarian stimulation could increase knowledge on what cellular mechanisms and pathways that are activated in response to the oestrogen surge. Proteomic studies could also reveal potential biomarkers.

The multiple comparisons necessitate correction to decrease the significance level and thus the number of false positives, but this comes with an increased risk of type 2-errors with increased number of false negatives and a risk that there could be hidden potential biomarkers among missed detection of significant protein change.

4.3 STUDY III – THE OBSERVATIONAL STUDY

4.3.1 Results

Characteristics of the patients in the observational study are presented in table 6 with baseline data. The women in the ART groups comprised 3.5 % of the study population. The women in

the fresh ET group comprised 25,382 women and 4,946 women comprised the frozen ET group. Women in the ART group were approximately six years older than women with spontaneous pregnancies. The women in the ART groups also had a higher educational level than women with spontaneous pregnancies and a lesser proportion of women with a low educational level, i.e. nine years or less.

Table 6. Baseline data of the observational study population. Reprinted with permission from the publisher under the Creative Commons CC-BY-NC-ND license [157].

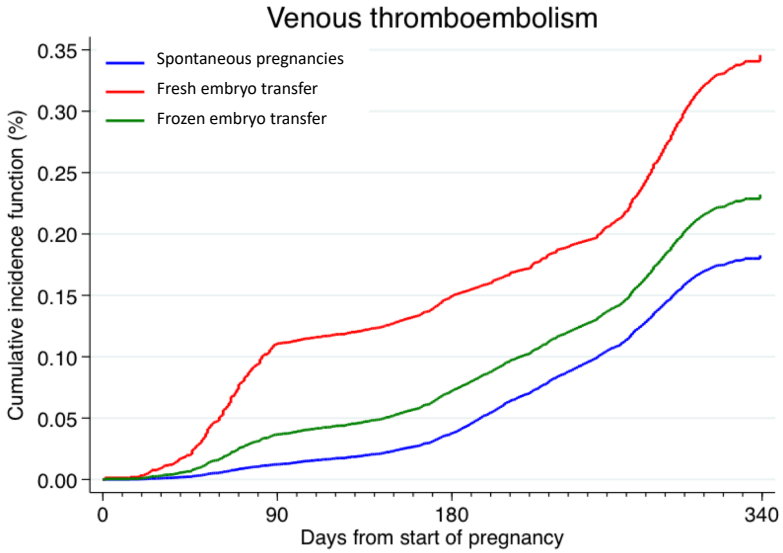
	Fresh ET[a]	**Frozen-thawed ET**	**Spontaneous**
Women, n	25,382	4,946	872,563
Median age, years [IQR] [b]	33.6 [30.8-36.5]	34.1 [31.2-36.9]	28.0 [24.6-31.3]
	Percentage, % and number (n) of women in each group		
Age, years			
<25	1.8 (466)	1.4 (68)	27.3 (238,417)
25-29	17.7 (4,486)	14.7 (727)	38.0 (331,216)
30-34	43.2 (10,955)	42.1 (2,084)	25.8 (225,431)
≥35	37.3 (9,475)	41.8 (2,067)	8.9 (77,499)
Year of delivery			
1992-1996	11.7 (2,959)	7.0 (345)	24.4 (213,078)
1997-2002	24.9 (6,308)	12.4 (615)	25.0 (218,199)
2003-2007	28.4 (7,218)	30.6 (1,513)	24.2 (211,552)
2008-2012	35.1 (8,897)	50.0 (2,473)	26.3 (229,734)
Country of birth			
Sweden	85.2 (21,615)	84.1 (4,158)	82.4 (719,375)
Other	14.8 (3,767)	15.9 (788)	17.6 (153,188)
Smoking status			
Yes	4.3 (1,121)	3.0 (148)	10.5 (91,153)
No	89.8 (22,726)	91.4 (4,521)	84.3 (735,748)
Missing	5.9 (1,535)	5.6 (277)	5.2 (45,662)
Body mass index, kg/m²			
<25	57.6 (14,619)	59.8 (2,956)	61.5 (536,054)
25-29	22.2 (5,633)	21.6 (1,067)	18.3 (160,031)
≥30	8.2 (2,082)	8.1 (402)	7.2 (62,685)
Missing	12.0 (3,048)	10.5 (521)	13.0 (113,793)
Education, years			
≤9	8.8 (2,237)	8.8 (433)	17.1 (149,482)
10-12	43.7 (11,085)	42.7 (2,112)	46.9 (408,707)
>12	46.9 (11,908)	48.1 (2,380)	34.0 (296,720)
Missing	0.6 (152)	0.4 (21)	2.0 (17,654)
Single birth	87.5 (22,201)	92.3 (4,566)	98.9 (862,844)
Multiple births	12.5 (3,181)	7.7 (380)	1.1 (9,179)

[a]ET=embryo transfer, [b]IQR=interquartile range

The proportion of smokers were higher in women with spontaneous pregnancies. BMI did not to differ markedly between the groups.

We found an increased incidence of VTE during all pregnancy including nine weeks postpartum after fresh ET with 4.7 per 1,000 pregnancies (n=119) as compared to 2.0 per 1,000 (n=1,760) in spontaneous pregnancies (HR 1.74, 95 % CI 1.43-2.12).

Figure 14. Cumulative incidence of venous thromboembolism after fresh (n=25,382) respectively frozen embryo transfer (n=4,946) and after spontaneous conception (n=872,563). Reprinted with permission from the publisher under the Creative Commons CC-BY-NC-ND license.[158].

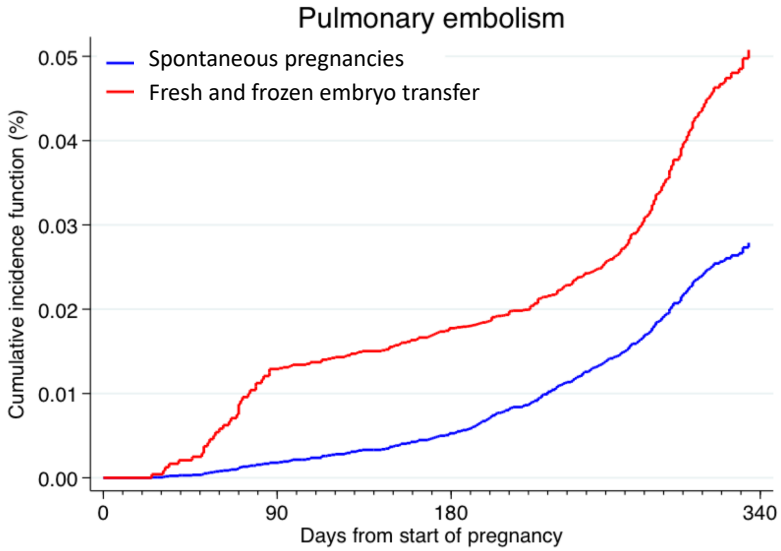


During the first trimester the incidence of VTE was 1.8 per 1,000 (n=45) as compared to 0.14 per 1,000 in spontaneous pregnancies (HR 8.96, 95 % CI 6.33-12.67). In table 7 the hazard ratios for VTE during pregnancy are presented for the entire pregnancy, including 6 weeks postpartum and by trimester.

Besides the overall VTE events, we also analysed the first incident PE during the first trimester in each woman separately and found an incidence of 0.32 per 1,000 (n=8) after fresh ET as compared to 0.03 per 1,000 (n=23) in spontaneous pregnancies (HR 8.69, 95 % CI 3.83-19.71), figure 15.

In table 7 the hazard ratios for VTE during the entire pregnancy including postpartum and by trimester are presented and in table 8 the hazard ratios for pulmonary embolism.

Figure 15. Cumulative incidence of pulmonary embolism after fresh and frozen embryo transfer together (n=30,382) and after spontaneous conception (n=872,563). Reprinted with permission from the publisher under the Creative Commons CC-BY-NC-ND license [158].



4.3.2 Discussion

We found an increased incidence of VTE in women undergoing ART with fresh ET as compared to women with spontaneous pregnancies. This was most prominent during the first trimester with a more than eight-fold increased incidence of VTE as well as PE compared to the first trimester in women with spontaneous pregnancies. These results show similarities to the findings of Henriksson *et al* who found an increased incidence in ART-pregnancies as compared to spontaneous pregnancies. Thus, the increased incidence during ART pregnancies during the first trimester seems to be confined to fresh ET.

We hypothesise that the reason explaining this finding is the ovarian stimulation with its oestrogen surge. The group of women with frozen ET had no increased incidence of VTE although the curve of the cumulative incidence could give an expression of a slight increase as compared to spontaneous pregnancy. One could speculate on the reason if this is a true phenomenon. One reason could be that infertility per se could increase the risk of VTE. Another reason could be that the hormones given in a "programmed" cycle during frozen ET could have an influence.

Table 7. Hazard Ratios for venous thromboembolism during pregnancy (including postpartum) and by trimester. Reprinted with permission from the publisher under the Creative Commons CC-BY-NC-ND license.

	Entire pregnancy (including postpartum)	**1st trimester[a]**	**2nd trimester[b]**	**3rd trimester[c]**	**Postpartum[d]**
Spontaneous (n=872,563)					
Events / incidence * (95% CI)	1760 / 2.02 (1.92-2.11)	126 / 0.14 (0.12-0.17)	264 / 0.30 (0.27-0.34)	640 / 0.74 (0.68-0.79)	730 / 0.84 (0.78-0.90)
Crude HRs (95% CI)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Multivariable-adjusted HRs§ (95% CI)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Fresh ET (n=25,382)					
Events / incidence * (95% CI)	119 / 4.69 (3.89-5.61)	45 / 1.77 (1.29-2.37)	16 / 0.63 (0.36-1.03)	19 / 0.75 (0.45-1.18)	39 / 1.54 (1.10-2.11)
Crude HRs (95% CI)	2.38 (1.98-2.87)	12.29 (8.74-17.27)	2.07 (1.25-3.43)	0.79 (0.50-1.25)	2.67 (1.94-3.69)
Multivariable-adjusted HRs§ (95% CI)	1.74 (1.43-2.12)	8.96 (6.33-12.67)	1.51 (0.91-2.51)	0.59 (0.37-0.93)	1.93 (1.39-2.68)
Frozen ET (n=4,946)					
Events / incidence * (95% CI)	17 / 3.44 (2.00-5.50)	3 / 0.61 (0.13-1.77)	3 / 0.61 (0.13-1.77)	6 / 1.22 (0.45-2.65)	5 / 1.01 (0.33-2.36)
Crude HRs (95% CI)	1.72 (1.07-2.78)	4.20 (1.34-13.20)	1.99 (0.64-6.22)	1.23 (0.55-2.75)	1.80 (0.75-4.33)
Multivariable-adjusted HRs§ (95% CI)	1.22 (0.76-1.98)	2.97 (0.94-9.35)	1.41 (0.45-4.41)	0.89 (0.40-1.99)	1.26 (0.52-3.04)

*=venous thromboembolisms per 1,000 ongoing pregnancies, §=adjusted for: age at delivery (<25, 25-29, 30-34, ≥35 years), pre-pregnancy BMI (<25, 25-29 or ≥30 kg/m^2), educational level recorded as number of school years (≤9 years -compulsory school, 10-12 years - upper secondary school, >12 years - university level), pre-pregnancy cigarette smoking status (yes/no), country of birth (Sweden or other country), and single versus multiple delivery.

[a] =day 0 to day 90 of pregnancy, [b] =day 91 to day 181 of pregnancy, [c] =day 182 of pregnancy until 3 days before the delivery date, [d] =2 days before the delivery date until 6 weeks after delivery date.

HR=Hazard Ratio, CI=Confidence Interval, ET=Embryo Transfer.

Table 8. Hazard Ratios for pulmonary embolism during pregnancy (including postpartum) and by trimester. Reprinted with permission from the publisher under the Creative Commons CC-BY-NC-ND license.

	Entire pregnancy (including postpartum)	**1st trimester[a]**	**2nd trimester[b]**	**3rd trimester[c]**	**Postpartum[d]**
Spontaneous (n=872,563)					
Events / incidence * (95% CI)	327 / 0.37 (0.34-0.42)	23 / 0.03 (0.02-0.04)	45 / 0.05 (0.04-0.07)	87 / 0.10 (0.08-0.12)	172 / 0.20 (0.17-0.23)
Crude HRs (95% CI)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Multivariable-adjusted HRs§ (95% CI)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Fresh ET (n=25,382)					
Events / incidence * (95% CI)	20 / 0.79 (0.48-1.22)	8 / 0.32 (0.14-0.62)	1 / 0.04 (0.00-0.22)	2 / 0.08 (0.01-0.29)	9 / 0.35 (0.16-0.67)
Crude HRs (95% CI)	2.17 (1.38-3.41)	11.96 (5.35-26.74)	0.76 (0.11-5.55)	0.51 (0.13-2.06)	2.71 (1.38-5.31)
Multivariable-adjusted HRs§ (95% CI)	1.58 (0.98-2.55)	8.69 (3.83-19.71)	0.56 (0.08-4.05)	0.38 (0.09-1.54)	1.97 (0.99-3.92)
Frozen ET (n=4,946)					
Events / incidence * (95% CI)	4 / 0.81 (0.22-2.07)	0 / —[e]	2 / 0.40 (0.05-1.46)	1 / 0.20 (0.01-1.13)	1 / 0.20 (0.01-0.13)
Crude HRs (95% CI)	2.19 (0.82-5.86)	—[e]	7.85 (1.90-32.34)	1.26 (0.18-8.98)	1.54 (0.22-11.03)
Multivariable-adjusted HRs§ (95% CI)	1.55 (0.57-4.19)	—[e]	5.51 (1.33-22.90)	0.90 (0.13-6.47)	1.08 (0.15-7.79)

* pulmonary embolisms per 1,000 ongoing pregnancies,§ adjusted for: age at delivery (<25, 25-29, 30-34, ≥35 years), pre-pregnancy BMI (<25, 25-29 or ≥30 kg/m^2), educational level recorded as number of school years (≤9 years -compulsory school, 10-12 years - upper secondary school, >12 years - university level), pre-pregnancy cigarette smoking status (yes/no), country of birth (Sweden or other country), and single versus multiple delivery.

[a] day 0 to day 90 of pregnancy, [b] day 91 to day 181 of pregnancy, [c] day 182 of pregnancy until 3 days before the delivery date,[d] 2 days before the delivery date until 6 weeks after delivery date, [e] Not computed due to zero pulmonary embolisms

HR: Hazard Ratio, CI: Confidence Interval, ET: Embryo Transfer

We found around 20 % PE respectively 80 % DVT in all of the groups, which is similar to previous findings in pregnancy.

Our aim was to study first trimester VTE, but in our material we also found an increased incidence of VTE in the postpartum period after fresh ET, which was not found in the research group's previous study [83]. We can only speculate why. Other Swedish studies on ART pregnancies have found that ART was associated with a higher incidence of caesarean section as delivery method and caesarean section is a known risk factor for VTE [159]. We have, however, not analysed an influence of delivery method in our material.

A strength of the study is that it is a nationwide study with a large material that included almost all women with deliveries in Sweden due to high coverage of the MBR. The study is an observational, registry-based study analysed in retrospect. However, the data analysed were prospectively collected, which is a strength.

One limitation of the study is that we lack information on treatment protocols and anticoagulant treatment. There could for example be a difference within the group of frozen ET between those given a hormone modified "programmed" cycle using exogenous hormones including mild stimulation by exogenous oestrogen as compared to a natural cycle.

Our study could be biased by a so called confounding by indication, which can be seen as a type of selection bias where the very reason for the infertility could be the reason for the increased adverse outcome, VTE. Thus, women in the ART group could as a whole be more prone to VTE, which might explain a part of the difference as compared to spontaneous pregnancies. However, the increase in the incidence of VTE in the fresh ET group is of a clearly higher magnitude than the previous speculation of a slight increase after frozen ET.

There is a risk of misclassification regarding the VTE diagnoses. There are studies that have shown quite discouraging results regarding VTE diagnoses in the Swedish inpatient registry where a positive predictive value (PPV) was 75 % [95], similar to previous Danish studies [139 160], but they were not performed specifically on pregnant women. In Denmark a study was performed on PPV for diagnose code in pregnancy and postpartum and they found a better PPV of 87 %, but which varied within diagnose coding and not all events had occurred during pregnancy or postpartum period. The clinical challenge and thus the strive for a correct diagnose in pregnancy might in the future result in more accurate diagnose codes of VTE.

A further bias in our study is the immortality time bias by study design and its population, i. e. the women included had to survive until child birth and we thus lack information on pregnant women who deceased during the pregnancy due to VTE or PE.

Regarding confounding factors we adjusted for age, BMI, multiple birth, smoking, calendar period of diagnosis, educational level as a proxy for socioeconomic status and country of birth. There is always the possibility of residual confounding after adjustment and of other risk factors not accounted for that affect both exposure and outcome such as OHSS,

inflammatory disease, previous cancer, thrombophilia, family history, anticoagulant treatment or dosing of exogenous hormones used.

OHSS might contribute to the observed risk. Rova *et al* found an incidence of 6-7 % OHSS and in the group of OHSS and fresh ET the VTE incidence was profoundly increased as compared to women with spontaneous pregnancies. Villani *et al* in their small cohort of 234 clinical pregnancies after ART, found 4 % OHSS, where only 30 % were prescribed thromboprophylaxis and none in that group had an event. Caveat that this was a very small, non-significant material with few events.

In the ART group you should also consider a possible so called "healthy woman effect" which in turn could lead to an underestimation of the risk.

In our previous study the groups were matched on age and calendar period. In the present study we had three groups, making matching a bit more complex since we aimed to compare two different exposures, fresh ET and frozen ET, with the same reference group. Thus we kept all spontaneous pregnancies after exclusion of the exposed groups and used them as a reference group. Yet, we did also perform a matched study analysis, as a sensitivity analysis, with the same Cox regression models where each woman in the total ART group (fresh and frozen ET) was matched on age with five controls in the group of spontaneous pregnancies without a change of the results presented. In figure 16 and figure 17 the graphs after age-matching are presented.

Figure 16. Age-matched cumulative incidence of venous thromboembolism after fresh (n=25,382) respectively frozen embryo transfer (n=4946) and after spontaneous conception (n=872,563). Not published.

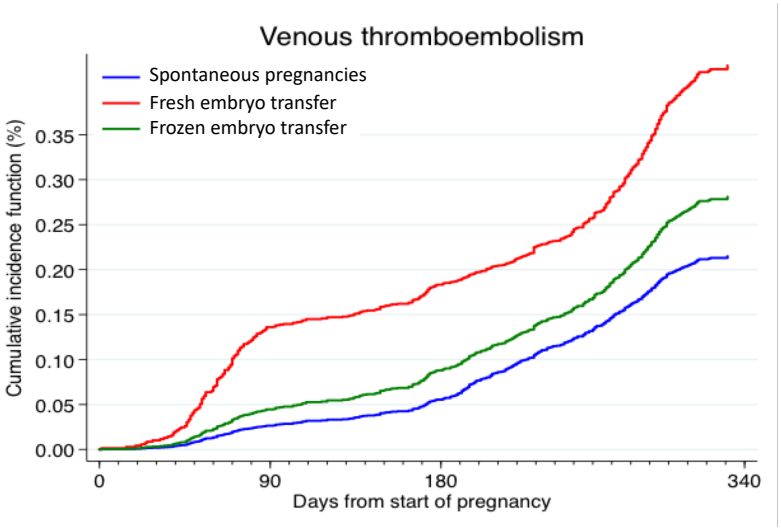
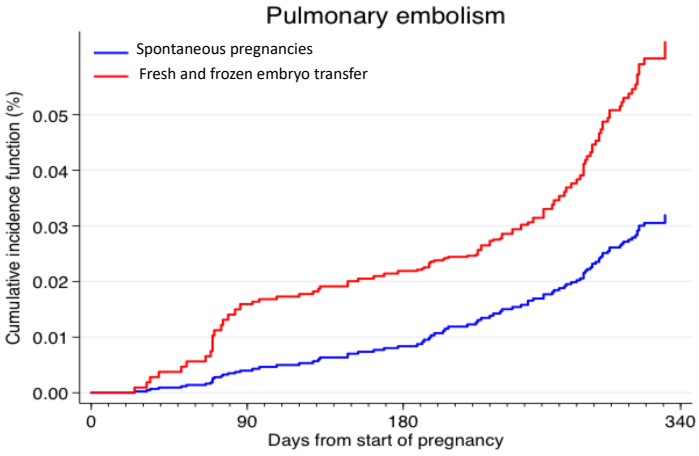


Figure 17. Age-matched cumulative incidence of pulmonary embolism after fresh and frozen embryo transfer together (n=30,382) respectively after spontaneous conception (n=872,563). Not published.



5 CONCLUSIONS

- Successful ART with fresh embryo transfer is associated with an increased incidence of VTE and PE during pregnancy and postpartum, mostly accounted for by a more than eight-fold increase during the first trimester, compared to spontaneous pregnancies.

- ART with ovarian stimulation increases the levels of circulating MVs with PMVs and EMVs expressing activation markers, indicating a hypercoagulable and proinflammatory state during ovarian stimulation.

- Proteomic analysis of MVs revealed alterations in proteins related to coagulation and complement activation during ovarian stimulation in ART indicating a hypercoagulable and proinflammatory state. We also found alterations of proteins related to reproduction.

6 GENERAL DISCUSSION

The absolute risk of VTE during pregnancy is indeed low. This applies to spontaneous pregnancies as well as to pregnancies after assisted reproduction. Despite a low incidence we clinicians should do our utmost to prevent this occurrence since VTE can cause severe morbidity and even mortality. Risk awareness and assessment is of great importance in order to perform ART with minimised risk and preferably without decreasing the chances of conceiving a child.

There might be different reasons for the increased incidence of VTE found in the first trimester and postpartum. Ovarian stimulation might be a plausible explanation during the first trimester and perhaps the method of delivery, caesarean section, during the post-partum period.

There is a tendency in the VTE curve of frozen ET in figure 14 to be increased as compared to spontaneous pregnancies, but this putative increase is not statistically significant. If this should be shown to be true in a larger study this might be explained by confounding by indication, that infertility might affect the risk of VTE. Furthermore, some women will be treated with oestrogen in a “programmed” cycle before frozen ET which could also contribute to an increased incidence of VTE.

The result of our observational study could speak in favour for a freeze-all strategy performing embryo transfer when the woman is not hyperstimulated by hormones and with the concomitantly increased levels of procoagulant microvesicles and up-regulated coagulation factors.

Another way to handle the risk is to use adequate thromboprophylaxis for pregnant women at risk of VTE, with or without ART. This is a way to be able to perform ART without having to compromise too much on which method or protocol would be best suited to achieve a pregnancy. Women at high risk of VTE before pregnancy should probably start thromboprophylaxis already at the start of the ovarian stimulation and if OHSS develops, immediately when it is diagnosed. Severe OHSS does indeed carry a major increase in VTE-risk, but it does not explain the whole risk burden after ART procedures. More studies are needed to identify women at risk of hyperstimulation as a cause of VTE. Studies comparing different treatment protocols, repeated testing of oestrogen levels and procoagulant and proinflammatory markers and studies on potential biomarkers are warranted.

7 FUTURE PERSPECTIVES

An increasing number of ART treatments are performed each year in Sweden and ART treatment have changed during the years combining an increased possibility of a successful pregnancy with a minimized risk of both VTE and obstetric complications.

Risk assessment and strategies for women with known VTE-risk during pregnancy and ART demand a close cooperation between several specialists. Obstetricians and gynaecologists together with coagulation specialists have to customise individual treatment to minimise the risk. In Sweden such a working group on haemostatic disorders (Hem-Arg) within the Swedish Society of Obstetricians and Gynaecologists (SFOG) have developed Swedish guidelines and a scoring system for thromboprophylaxis during pregnancy. These recommendations even cover ART and when to start thromboprophylaxis depending on risk assessment, including risk of OHSS.

As mentioned, since 2007 the specialised Q-IVF registry has gathered information on almost all ART treatments from the fertility clinics in Sweden. With the increasing numbers in the registry of both fresh and frozen ETs performed, the power of registry data from Q-IVF to perform direct comparisons regarding the VTE-risk of the two methods will increase. The addition of biomarkers could also help to identify when to use thromboprophylaxis.

Our proteomic analysis revealed alterations of proteins related to reproduction. Future studies will show whether any of these proteins could be used as biomarkers of a successful ART. Women will have to be followed until successful pregnancy and correlation studies could identify putative biomarkers. This would also necessitate future larger and repeated proteomic studies. Those could contribute to the understanding of the pathways involved and identification of biomarkers of successful pregnancy in ART and of risk of diseases such as VTE.

Hopefully future ART treatments will be associated with an increased number of successful pregnancies performed with a minimised risk of VTE.

8 SVENSK SAMMANFATTNING

Venös tromboembolism (VTE) är ett samlingsbegrepp för blodproppar i de djupa venösa kärlen och delar av proppar som har lossnat och färdats med cirkulationen, vanligen till lungans kärl. Det kallas djup ventrombos (DVT) och lungembolism (LE). Under graviditet drabbas 1-2 av 1000 kvinnor av VTE och man har i tidigare studier sett att risken var ännu större vid assisterad reproduktionsteknologi (ART), så kallad ”provrörsbefruktning”. Den största skillnaden har man sett under den första tredjedelen (trimestern) av graviditeten där sju gånger fler kvinnor drabbades av lungemboli under ART-relaterade graviditeter jämfört med spontana graviditeter. Användningen av ART ökar kontinuerligt och i Sverige i dag föds mer än 4 % av alla barn efter assisterad befruktning.

Målet med studierna i doktorandprojektet var att öka kunskapen om möjliga mekanismer som kan förklara att fler kvinnor drabbas av VTE efter ART jämfört med spontan graviditet. Det bör i sin tur öka förståelsen för hur risken för VTE kan minimeras. Eftersom skillnaden var som störst i den första trimestern är vår hypotes att det är hormonstegringen vid äggstocksstimulering under ART som är orsaken till ökningen.

Vi skilde därför på ART med färsk embryoåterföring, dvs när graviditeten skett genom embryoåterföring direkt efter den fas då kvinnan erhållit behandling med äggstocksstimulering, respektive senarelagd embryoåterföring av ett fryst och tinat embryo. Vi jämförde hur många som drabbades av VTE och LE i de två grupperna med hur många som drabbades vid spontan graviditet. Vi såg att mer än åtta gånger fler drabbades av VTE och specifikt av LE i gruppen med färsk embryoåterföring jämfört med spontana graviditeter. Skillnaden var som störst under den första trimestern, men en ökning sågs också under hela graviditeten och under de första sex veckorna efter förlossningen. I gruppen med frystinad embryoåterföring sågs ingen signifikant ökning jämfört med spontana graviditeter.

Vi studerade också effekten av äggstockstimuleringen på mikrovesiklar och deras proteininnehåll. Det gjorde vi genom att undersöka insamlade prover från 31 kvinnor. Proverna var tagna före äggstocksstimuleringen när östrogen-nivåerna var nedreglerade och under äggstocksstimuleringen när östrogen-nivåerna ökat 10-100-faldigt. Vi såg en ökning av mikrovesiklar som indikerade ett prokoagulativt tillstånd med tecken till trombocytaktivering som både skulle kunna vara bidragande till en ökad proppbenägenhet och markörer för en ökad VTE-risk. Vi studerade även proteininnehållet i mikrovesiklarna och såg en förändring av flera proteiner som bidrar både till koagulation och inflammation samt proteiner relaterade till reproduktion.

Sammanfattningsvis är det ovanligt att drabbas av VTE och LE, men man ser en ökad andel kvinnor som drabbas under graviditet såväl med som utan assisterad befruktning. Eftersom VTE medför en risk för långsiktiga komplikationer och VTE och lungemboli är orsaker bakomliggande mödradödlighet är det förstås av yttersta vikt med fortsatta studier för att

uppnå en ytterligare ökad förståelse för de bakomliggande mekanismerna för att därigenom kunna möjliggöra en minskad risk.

Det är av stor vikt med fortsatt tvärprofessionell forskning och samarbeten mellan specialister inom kvinnors hälsa och reproduktion och specialister verksamma inom området koagulationssjukdomar.

9 ACKNOWLEDGEMENTS

I feel blessed to have and have had so many important persons in my life and I cannot thank you all enough, but I will take the chance to express some of my gratitude here.

My main supervisor, Peter Henriksson. You have been a fantastic supervisor, including me in all parts of the research and your life. All the meetings you thought I could learn something from that you included me in, also in your other or future projects so I could be part from the very start of planning new research. You see beyond the doctoral project and made me feel important. You have taught me a lot about all details on how to perform research and always with an ethical and I would say correct way, the way research of course always should be performed. Your curiousity of other people is something I appreciate and something I share with you. Your knowledge and intelligence is impressive. Thank you so much for your company these years, your patience and respect for me and other people, your support in the other parts I engage in at work, the important residents, our ST-doctors, the clinical work and of course my most important engagement in my children Tristan and Mika. Mika, who was born in the beginning of the project in 2014. I hope we can go to a hockey game together some day, yet in different colours. Heja Löven!

My co-supervisor Fariborz Mobarrez, you have always been available for me and explained some of the most difficult parts of the project to me, a world of molecules I thought I left after KIs pre-clinical exam, but you made me curious in again. You have such pedagogic skills, the Uppsala students are truly blessed! Thank you for always being so warm and heartly and positive about everything. You always made me in a good mood and supported me both directly and in a subtle way when I think I needed it more than I realised, when I focused on my own limitations too much.

My co-supervisor Katarina Bremme, I am so glad you accepted to participate in the project. To have an expert like you on women's health and reproduction in the project is of such a huge importance. You are a true role model and your engagement in the patients, your curiousity, your knowledge, your research and your hard work is impressive! Finding you at the congress in Madrid made me so happy – you are such a great company!

My co-supervisor Anders Ekbom, the true expert on epidemiology research. Having you as part of this project's observational study was of great importance. I must thank you also for being the founder of the Research school for clinicians in epidemiology which is fantastic.

Mentor Anna Ågren, I have always felt that you are ready to catch me when I fall. If I fall. And to have that feeling, that you are there for me, has meant a lot. You always have the best clinical and private counselling advise based on your deep knowledge and wise personality and the clarity of your answers is unique.

Eli Westerlund, my informal extra-extra-supervisor-mentor. You are a superwoman. I can truly not ever understand how you can manage to perform so well in so many areas. I wanted you to be our future boss at the clinic or why not the whole hospital, but it is so good that you engage in research and the clinic where you are so needed. You are a coach, a supporter, a teamplayer and I don't know if you have any limitations. You are inspiring. Thank you for the co-work in resident courses, the laughs, the tears brought out from your son Emil's beautiful singing. I look forward to growing old together at Danderyd Hospital.

Eva-Lotta Hempel, I will never stop missing you at Danderyd Hospital. And I am not alone. We will always try to win you back. You and Hans Johnsson with your shared passion and skills in the field of coagulation made me passionate as well. Eva-Lotta, you took me through my residency, helped me get to Karolinska Coagulation Unit. Your competence and engagement in the patients at ward 75 and Trombosmottagningen together with Linda Paulsson back then (now our Linda Löf who in a brilliant way took your place at ward 75 IMA) was always inspiring and admirable. The patients we have kept still miss you. You are so supportive and engaged. To have you in the woods when Tristan hurt his back and lost his breath felt to me like you are my guardian angel wherever.

Andrea Discacciati, my other informal co-supervisor. I never found the way to your room at IMM, but you always escorted me so kindly with the same patience you escorted me through the complex statistical considerations. Our many sessions planning the analysis, performing it and the weeks we spent thinking and discussing some of the results. Many daily e-mails some periods that I have rediscovered now before defending the thesis. You taught me a lot and I had a lot of fun with you there and seeing you at the ghost castle, one of the most scary nights I have spent and I don't believe in ghosts. Thank you again for guiding me in the world of biostatistics where Matteo Bottai gave us the theory at the Research school.

Håkan Wallén who has been such a perfect mentor for me as a resident, as a PhD candidate and at the Haemostasis centre. You always see to everyones good and you are always so helpful. I never felt that you didn't have the time for my questions although you had far too much to do at work at the clinic and for KI. You also participated in this research and our discussions at research meetings always gave me a lot to think about. Fika at the Haemostasis Centre is always the best when you are around.

Haemostasis Centre, where I gained so much knowledge on VTE and the clinical field of coagulation. You who opened up my eyes are Eva-Lotta Hempel and Hans Johnsson, Anders Carlsson and Anna Arctaedius at the start. And Berit Selander who helped me so much in the beginning when I was the only and lonely doctor there. Working with you all now is great, Per Sandén kind and competent, you cardiologists who teach me so much, Maja Andersson, Bruna Gigante, Kristina Hagwall and Mika Skeppholm. And skilled Cecilia Hedlund, Anita Englund, Markus Olsson, Madelene Sellman and Indika Tourde. Still miss you Maja Asperen and hope to join your yoga class some day. And Lisa Andersson and Hillevi Käll with your positive energy and our private talks. Dawit who also always makes me in a good mood.

One thing I have learnt is that many people contribute to every research article published. A lot of work is behind all research and my respect has grown for the many details, in planning the research, writing ethical applications, writing funding applications, preparing the data, performing statistical analysis, spending a day on a graph, etc. I would especially like to thank Frida Lundberg and Anastasia Iliadou Nyman with whom we had early meetings, planning and discussing the observational study. You shared your competence and Frida prepared the data so well by combining the registries.

I would of course like to thank my closest little family, Kalle, Mika and Tristan. You are the best! The love I feel for you is enormous and I admire and appreciate your patience with me this year through all work during the first months of the pandemic and this fall preparing my thesis and dissertation.

My *allra käraste syster* Anna. Words. Words are important. Trying to express love is important. But the love I feel for you and the connection we have is beyond description. You know. I am so touched by your engagement in this thesis during my last stressful weeks before printing. You grasped it all about coagulation, microvesicles and venous thromboembolism, but then you are also the brightest one I know and wisest. You and Yannis always make me feel at home. I love you both deeply.

My mother Inger and my father Rolf. As I sang to Mika and Tristan "*Vad vore jag utan era andetag*". You are my role models. My father, always one of the most appreciated persons at work and in private contexts. With a justice pathos, which probably made you the best legal adviser and most certainly the best father. My mother, the most social, the most patient person I know, the most appreciated teacher I've heard of. You changed people's lives. And you are the most loving mother and grandmother. A bit of a curler. I love you for that. And I love travelling with you both and I long for the next possible trip to Bilabo and I dream of our old trips to Sarek and Italy.

Marie Alexandra, agapi mou gia panda. You are always there for me and you contributed a great deal even to my doctoral project by taking care of Mika twice in Barcelona when I presented posters. I love you for that and for all the fun we have had and will have. Ingegerd och Uno were, and I am so proud of you and Leon and Malkom. Your parents would love your new big family with Abbe, Leona and Jonathan.

My double-cousin-sisters Maje and Sofia. Double-cousins are the best, an extra couple of beloved sisters. And your parents an extra-couple of parents, Inga-Britt and Thore. I love you all so much. You are always there for me and we for each other. Your homes are my homes. Having you and moster Bettan and moster Anne & Robban is having a lot of extra homes.

Samme, brother from another mother, "*Avsaknaden av evidens innebär inte att det finns evidens för motsatsen.*". Våra hetsiga diskussioner har förgyllt mitt liv.

My big, beloved, "*trygga*" family and friends. I miss you all so much and love in the time of a pandemic is hard. I dream about seeing you all in a gathering, you know who you are, in

farmors Pempelijärvi, mormors Kainulasjärvi, Baskien, Berlin, Iraklion, Norrköping, Forsnäs, Sörål, Nyhyttan, at a Björklöven hockey game or perhaps at a big gathering in Toscana.

My mother-in-law Lotta, I would have loved to reach the goals of our respective research together. Most of all I would have loved that you lived to spend all your love on your grand children. We talk about you a lot and Tristan and Mika knows and can feel your love.

The parents of my children´s best friends. You are so kind and your children the best. Thank you for also helping me get time to finish this thesis, among you Bo, Lena, Lisa, Markus, Marie and Sara.

I have so many colleagues to mention, afraid to forget someone important. Thank you to the department of Medicine that makes research possible in our clinic by all help from Elin Rundqvist and Åsa Tolonen and trough decisions by our former heads of department, Andreas Kling, Mats Söderhäll and Karin Malmqvist and my closest superiors and close colleagues during these years Rebecca Undén Göransson, Johan Ejerhed and Emilie Asplund. Rebecca, Johan and Emilie – you always seemed so happy and positive there at ward 76 so I had to join you. You are a great gang, ward 76/98-colleagues, all of you colleagues in all professions! I go to work smiling because of the engagement and the collegial environment we all have there!

All you soon-professors Sara Tehrani, Charlotte Thålin, Annika Lundström and docent Lotta Laska. You are inspiring to the whole clinic and you keep up the scientific spirit. Lotta and Magnus von Arbin always kept the scientific approach, teaching us early residents about the most important clinical trials. Thank you. Annika, you are also someone I can talk to about everything, existential and relationship issues and you are really a warm person. I am happy to have you as a colleague ever since Dalens sjukhus. I am also happy we had dear Anna Haeggström with us from Dalen, who I frequently consult in her area of expertise.

Karin Skullman Lagerholm and Eleonore Närlinge, you contributed with new energy at different time points. Thank you for the inspiration and the discussions about new studies. Even if we didn't take all of them further you inspired to the ongoing and future research. And I learnt how to write ethics applications and planning of a new study with you, Karin. Perhaps we should take it all further in the future?

Gargi Gautam, you make me laugh so much and you are very good at creating an informal and relaxing environment. Simultaneously with being one of our most competent colleagues and coolest doctors. I am impressed with your research work and happy to have you as a colleague at the clinic and most of all as a colleague friend at the Haemostasis Centre!

Jacob Philipson, you left us for the West Coast. But I do hope you will be part of the Coagulation unit in Gothenburg and continue your work on VTE during pregnancy, so we can meet at congresses and workshops in the future.

Louise Ziegler and Anna Grünfeldt, my room mates and now impressingly two of the most important persons at our clinic. I am so happy to have you as my colleague friends and room mates. One can truly rely on you and you both are great listeners. We have so many close and rewarding discussions. Anna, stay in the room! Louise, I will hang out a lot in your room!

Thank you to all the great colleagues at our department who contribute to the great atmosphere we have. All residents at our Department of Medicine - I really enjoy being your "studierektor". You are all so engaged and the most hard working colleagues at our clinic I would say. The years of union work were also important to me. Thank you Ellinor Skogseid, Peje Åstrand, Anette Hein and Johan Styrud who taught me a lot. And Samantha Rutherford Lörstad who keeps striving for our working conditions.

Camilla di Battista, I do hope we can be close colleagues some day. Until then close friends. You know all the studies. You know all about the smallest cultural events. You know all about the best restaurants. We need to go to Larry's corner soon.

Adriano Atterman, you took care of me when I fell, when my young cousin Alexander passed away. And after him the brothers Filip and Emil. I will never forget that. You made me laugh so much during research shool and after that with your great sharing of small joys and soft and mellow music tips. And I really needed that art walk exhibition. Thank you.

Johan Reutfors, our vice principal at the research school and our group of colleagues there who all created such a good atmosphere while learning scientific theory.

Thank you for always helping me wheen in need, Åsa Lilja and Nina Ringart.

Thank you for also opening up my heart and brain to the coagulation field of course Margareta Holmström, Aleksandra Antovic, Niklas Bark, Maria Bruzelius, Roza Chaireti, Maria Farm, Lars-Göran Lundberg, Mårten Söderberg and Cecilia Jennersjö, the most entertaining, sharing social media-contact I have. Special thanks to Margareta who has always been so generous, kind and sharing her super-competence. And final, special thanks to the most competent, experienced, engaged researcher Margareta Blombäck. I was so thrilled to once having the opportunity to spend time on a bus with you. I planned to ask you everything about coagulation, but ended up listening to you talking about literature and poetry. You are amazing.

You all have been an inspiration in research and life and finally I got the chance to thank so many of you. It's all about relationships as my beloved cousin Calle and my main-supervisor Peter use to say.

Tack!

The studies have been founded by the Swedish Heart-Lung foundation, by grants from the Swedish state under agreement between the Swedish government and the county councils, the ALF agreement, and local means of the Department of Medicine, Danderyd Hospital.

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